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**PURIFICATION, mRNA EXPRESSION
AND REGULATION OF PYROGLUTAMYL PEPTIDASE II**

by

JUN LIN

A dissertation submitted to the Graduate Faculty in Biomedical Sciences
in partial fulfillment of the requirements for the degree of Doctor of Philosophy,
The City University of New York

1997

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ABSTRACT

PURIFICATION, mRNA EXPRESSION AND
REGULATION OF PYROGLUTAMYL PEPTIDASE II

by

Jun Lin

Adviser: Professor Sherwin Wilk

We have purified the specific thyrotropin releasing hormone (TRH) degrading enzyme pyroglutamyl peptidase II from rabbit brain to apparent homogeneity as judged by SDS-PAGE and nondissociating gel electrophoresis. Its subunit structure was characterized as a homodimer with a native molecular mass of 230 kDa and a subunit molecular mass of 115 kDa. The specific enzymatic activity was estimated as 135 units/mg. The amino acid sequences of two tryptic peptides were obtained with the purified enzyme and they were found within the later reported cDNA sequence. The biochemical properties are consistent with a glycosylated membrane protein.

The distribution and quantitation of pyroglutamyl peptidase II mRNA in rat major organs and brain regions were determined by a reliable and sensitive solution hybridization ribonuclease protection assay. The highest level of pyroglutamyl peptidase II mRNA was found in brain. Of the brain regions studied, the highest level was found in posterior cortex. Significant levels of the mRNA were also found in lung and pituitary, whereas lower but detectable amounts were found in other peripheral organs. In rats treated with a single

dose of T₃, the level of pyroglutamyl peptidase II mRNA was markedly increased in the pituitary ($p < 0.01$), and in the liver ($p < 0.05$). A single dose of TRH had no significant effect on pyroglutamyl peptidase II mRNA levels. Elevation of pyroglutamyl peptidase II mRNA in liver by T₃ suggests that this organ is the source of the enzyme in serum.

The regulation of pyroglutamyl peptidase II mRNA was investigated in GH3 cells. TRH decreased pyroglutamyl peptidase II mRNA with a maximal effect seen 16h after exposure to 10^{-7} M TRH. The protein kinase C (PKC) activators phorbol-12 β -myristate-13-acetate (PMA), and phorbol-12,13-dibutyrate also decreased pyroglutamyl peptidase II mRNA levels, whereas the inactive phorbol ester analog 4- α -TPA had no effect. The protein kinase C inhibitor H-7 inhibited the decrease in pyroglutamyl peptidase II mRNA produced by TRH and PMA. Exposure to T₃ did not change pyroglutamyl peptidase II mRNA levels. EGF treatment (5×10^{-9} M for 5 days) produced a small (30 %) but significant increase in pyroglutamyl peptidase II mRNA in GH3 cells. The down-regulation of pyroglutamyl peptidase II mRNA by TRH may provide a positive feedback mechanism for the action of TRH on pituitary lactotrophs.

DEDICATION

To my wife, mother, father and brother.

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TABLE OF CONTENTS

Approval Page	ii
Abstract	iii
Dedication and acknowledgments	v
Table of contents	vi
List of figures	ix
List of tables	xiv
List of abbreviations	xvi
Chapter 1: Review of literature and significance of studies	1
1. Physiology and pharmacology of TRH	2
1.1. TRH as a hypothalamic-hypophysiotropic hormone	2
1.2. TRH as a transmitter/modulator	5
1.3. Pharmacological effects of TRH	7
2. Enzymology of TRH metabolism	8
2.1. Metabolic pathways of TRH	8
2.2. Properties of prolyl endopeptidase	9
2.3. Properties of pyroglutamyl peptidase I	10
2.4. Functional aspects of TRH degrading enzymes	11
3. Pyroglutamyl peptidase II	11
3.1 Serum thyroliberinase	11
3.2. Pyroglutamyl peptidase II in CNS	12
3.3. Regulation of pyroglutamyl peptidase II	15
3.4. cDNA clone of pyroglutamyl peptidase II	16
4. Aims of our studies	17

Chapter 2: Purification and characterization of subunit structure of	
pyroglutamyl peptidase II	20
Abstract	21
Introduction	22
Materials and methods	24
Results	29
Discussion	32
Figure Legends	35
Figure 1-22	42
Table 1-3	64
Chapter 3: Distribution and regulation of pyroglutamyl peptidase II	
messenger RNA levels in rats as determined by a quantitative	
ribonuclease protection assay	67
Abstract	68
Introduction	69
Experimental procedure	71
Results	77
Discussion	79
Figure Legends	84
Figure 1-6	87
Table 1-6	93
Chapter 4: Regulation of pyroglutamyl peptidase II mRNA in GH3 cells	99
Abstract	100
Introduction	101
Materials and methods	103

Results	106
Discussion	108
Figure Legends	111
Figure 1-22	114
Table 1-2	120
Chapter 5: Summary	122
Chapter 6: References	130

LIST OF FIGURES

<u>Figure</u>	<u>Page</u>
Chapter 1	
Figure 1. Metabolic pathways of TRH	9
Chapter 2	
Figure 1. Solubilization of pyroglutamyl peptidase II (PPII) by CHAPS.	42
Figure 2. Solubilization of pyroglutamyl peptidase II (PPII) by Triton X-100.	43
Figure 3. Solubilization of pyroglutamyl peptidase II (PPII) by deoxycholate.	44
Figure 4. Solubilization of pyroglutamyl peptidase II (PPII) by NP-80.	45
Figure 5. Solubilization of pyroglutamyl peptidase II (PPII) by Lubrol-px.	46
Figure 6. Dose-effect of trypsinization of pyroglutamyl peptidase II.	47
Figure 7. Time effect of trypsinization of pyroglutamyl peptidase II.	48
Figure 8. Sephadex G 300 chromatography of pyroglutamyl peptidase II following strong trypsinization (80 µg/ml trypsin at 37 °C for 1 hr) and acid precipitation.	49
Figure 9. Q-Sepharose chromatography of pyroglutamyl peptidase II following strong trypsinization (80 µg/ml	50

trypsin at 37 °C for 1 hr), acid precipitation, and
Sephadex G 300 chromatography.

- Figure 10. Phenyl Sepharose chromatography of
pyroglutamyl peptidase II following strong
trypsinization (80 µg/ml trypsin at 37 °C for 1 hr),
acid precipitation, Sephadex G300 chromatography
and Q-Sepharose chromatography. 51
- Figure 11. DEAE affi gel blue chromatography of
pyroglutamyl peptidase II followed a strong
trypsinization (80 µg/ml trypsin at 37 °C for 1 hr) acid
precipitation, Sephadex G 300, and Q-Sepharose
chromatography. 52
- Figure 12. Lentil lectin Sepharose chromatography of
pyroglutamyl peptidase II following strong
trypsinization (80 µg/ml trypsin at 37 °C for 1 hr) acid
precipitation, Sephadex G 300, and Q-Sepharose
chromatography. 53
- Figure 13. Q-Sepharose chromatography of pyroglutamyl
peptidase II following facile trypsinization (2 µg/ml
trypsin at 4 °C for 15 min). 54
- Figure 14. Sephadex G 300 chromatography of
pyroglutamyl peptidase II following facile
trypsinization (2 µg/ml trypsin at 4 °C for 15 min) and
Q-Sepharose chromatography. 55
- Figure 15. Lentil lectin Sepharose chromatography of
pyroglutamyl peptidase II following facile 56

trypsinization (2 $\mu\text{g/ml}$ trypsin at 4 $^{\circ}\text{C}$ for 15 min),
Q-Sepharose chromatography and Sephadex G
300 chromatography.

- Figure 16. Phenyl Sepharose chromatography of
pyroglutamyl peptidase II following a facile
trypsinization (2 $\mu\text{g/ml}$ trypsin at 4 $^{\circ}\text{C}$ for 15 min),
Q-Sepharose chromatography and Sephadex G 300
chromatography. 57
- Figure 17. Polyacrylamide gel electrophoresis (5% gel) of
pyroglutamyl peptidase II after the final step of
purification. 58
- Figure 18. SDS-PAGE (9% gel) of rabbit brain
pyroglutamyl peptidase II after the final step of
purification. 59
- Figure 19. The monomer molecular weight of pyroglutamyl
peptidase II. 60
- Figure 20. Estimation of the protein concentration of
pyroglutamyl peptidase II. Different concentrations
of β -galactosidase were used as standards. 61
- Figure 21. Western blot of the mouse antiserum against
purified pyroglutamyl peptidase II and the
corresponding band from a rabbit brain homogenate. 62
- Figure 22. Recognition by radioimmunoassay of
pyroglutamyl peptidase II by a mouse antiserum
generated by immunizing with purified rabbit enzyme. 63

Chapter 3

- Figure 1. Schematic representation of the pyroglutamyl peptidase II subclone for synthesis of riboprobe and standards. 87
- Figure 2. Relationship between volumes of hybridized bands and pg in vitro synthesized sense RNA as determined by a ribonuclease protection assay. 88
- Figure 3. Pyroglutamyl peptidase II mRNA in rat organs determined by a ribonuclease protection assay as described in Methods. 89
- Figure 4. Quantitation of pyroglutamyl peptidase II mRNA in rat brain regions, pituitary and spinal cord by a ribonuclease protection assay as described in Methods. 90
- Figure 5. Effect of T3 and TRH on pyroglutamyl peptidase II mRNA in rat pituitary and frontal cortex. 91
- Figure 6. Effect of T3 and TRH on pyroglutamyl peptidase II mRNA in rat liver and lung. 92

Chapter 4

- Figure 1. Pyroglutamyl peptidase II mRNA in GH3 cells as determined by a ribonuclease protection assay. 114
- Figure 2. Effect of TRH on pyroglutamyl peptidase II mRNA in GH3 cells as a function of the time of exposure. 115
- Figure 3. Dose- response effect of TRH on pyroglutamyl peptidase II mRNA levels in GH3 cells. 116

Figure 4. Effect of PMA treatment on pyroglutamyl peptidase II mRNA levels in GH3 cells as a function of time of exposure.	117
Figure 5. Dose-response effect of PMA on pyroglutamyl peptidase II mRNA levels in GH3 cells.	118
Figure 6. Effect of EGF on pyroglutamyl peptidase II mRNA levels in GH3 cells.	119

LIST OF TABLES

<u>Table</u>	<u>Page</u>
Chapter 2	
Table 1. Solubilization of pyroglutamyl peptidase II from rabbit brain by several detergents.	64
Table 2. Purification of pyroglutamyl peptidase II from rabbit brain using a strong trypsinization procedure.	65
Table 3. Purification of pyroglutamyl peptidase II from rabbit brain following facile trypsinization.	66
Chapter 3	
Table 1. Pyroglutamyl peptidase II mRNA levels and enzymatic activity in rat organs.	93
Table 2. Pyroglutamyl peptidase II mRNA levels and enzymatic activity in rat brain regions and spinal cord.	94
Table 3. Effect of T3 and TRH on pyroglutamyl peptidase II mRNA concentrations in rat organs.	95
Table 4. Effect of T3 and TRH on pyroglutamyl peptidase II mRNA concentrations in rat brain regions and spinal cord.	96
Table 5. Effect of T3 and TRH on pyroglutamyl peptidase II activities in rat organs.	97
Table 6. Effect of T3 and TRH on pyroglutamyl peptidase II activities in rat brain regions and spinal cord.	98
Chapter 4	
Table 1. Regulation of pyroglutamyl peptidase II mRNA levels in GH3 cells by TRH and PKC activators.	

Table 2. Effect of treatment of GH3 cells with 10^{-7} M T3
for varying peroids of time.

LIST OF ABBREVIATIONS

ADP, adenosine 5'-pyrophosphate

ATP, adenosine 5'-phosphate

BSA, *bovine serum albumin*

CPK, creatine phosphokinase

DMSO, dimethyl sulfoxide

DPP IV, dipeptidyl aminopeptidase IV

EDTA, ethylenediaminetetra-acetate

EGF, epidermic growth hormone

H7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine dihydrochloride

NA, naphthylamide

PAGE, polyacrylamide gel electrophoresis

PKC, protein kinase C

PMA, phorbol 12 β -myristate-13-acetate

PPII, pyroglutamyl peptidase II

SDS, sodium dodecyl sulfate

T3, 3',5'-triiodo-L-thyronine

TBS, Tris-buffered salient

TTBS, Tween 20 in Tris-buffered salient

TRH, thyrotropin-releasing hormone

Tris, 2-amino-2-hydroxymethyl-propan 1,3diol(tris(hydroxymethyl)methylamine)

Chapter 1

Review of Literature and Significance of Studies

Thyrotropin-Releasing Hormone (TRH) functions as a hormone, a putative neurotransmitter or modulator, and produces a variety of pharmacological effects. Studies on the properties of its degrading enzymes may not only contribute to an understanding of the physiology of TRH, but also may provide a basis for an alternative approach for pharmacological intervention.

1. Physiology and Pharmacology of TRH

1.1. TRH as a hypothalamic-hypophysiotropic hormone

TRH was first isolated as a hypothalamic-hypophysiotropic hormone functioning to promote the secretion of pituitary thyroid-stimulating hormone (TSH) (Schally and Redding, 1965; Schally, et al., 1969). Its chemical structure was characterized as the tripeptide L-pyroglutamyl-histidyl-prolylamide (Boler, et al., 1969; Burgus, et al., 1970). The synthesis of TRH and its analogs, greatly facilitated the development of antibodies against TRH, and radioimmunoassays for measurement of TRH (Bassiri and Utiger, 1972).

The dominant role of hypothalamic TRH in pituitary TSH synthesis and secretion has been well documented. The action of TRH on TSH release is dose-dependent and achieved at nanomolar concentrations (Vale, et al., 1972). Hypothyroidism and decreased TSH concentrations were observed following hypothalamic-pituitary dissociation and hypothalamic lesion (Greer, 1952; Jackson and Reichlin, 1977). TRH is present in the portal circulation at physiologically relevant concentrations (Scheward, et al., 1983).

In addition to stimulation of TSH release, TRH also stimulates the release of prolactin (Jacobs, et al., 1971; Takahara, et al., 1974). This action is as potent as its TSH releasing effect (Noel, et al., 1974). In rats, secretion of prolactin in response to suckling and mammary stimulation was observed and was accompanied by TSH release and elevation of the TRH concentration in portal circulation (Burnet and Wakerley, 1976; DeGree and Visser, 1981). TRH antibodies can reduce the response of prolactin secretion to heat in sheep (Fraser and McNeily, 1983). However, TRH is not the sole mediator of prolactin secretion. The regulation of prolactin secretion is complex and subject to more dominant control by other hypothalamic factors such as dopamine and prolactin releasing factor (Harris, et al., 1978; Leong, et al., 1983). The physiological role of TRH in prolactin secretion in man is not clear, as stimulation which releases prolactin generally does not result in the release of TSH.

Under certain pathological conditions, TRH stimulates the release of other pituitary hormones such as growth hormone and ACTH (Review, Prasad, 1985). Under these conditions, the functional association between the hypothalamus and pituitary may be impaired, which may deviate the normal response of the pituitary hormones to their regulatory factors.

The actions of TRH on pituitary thyrotropes and lactotropes are mediated by high affinity and specific receptors. Specific binding sites in anterior pituitary for TRH were successfully revealed by use of the stable analog H^3 -MeHis-TRH as a radioligand (Hinkle, et al., 1974; Hinkle and Tashjian, 1984). The interactions of TRH with receptors are mediated via G proteins coupled by the phospholipase C pathway (Gershengorn, et al, 1980; Martin, 1983). Binding of TRH to receptors results in the stimulation of phosphoinositide turnover with an

increase in the cellular levels of two second messengers, diacylglycerol and IP₃ (Straub and Gershengorn, 1986). Diacylglycerol increases the cytosolic Ca²⁺ level, which accounts for the rapid phase release of prolactin and TSH, while the activation of protein kinase C by IP₃ accounts for the second phase of secretion (Gershengorn, 1986). The TRH receptor has been cloned and shown to belong to the large family of G protein coupled receptors (GPCRs) (Straub, et al, 1990).

The hypothalamic-pituitary-thyroid axis is under strict regulation. Thyroid hormone can inhibit the effect of TRH on TSH release, but this modulatory action of thyroid hormone can be overcome by large doses of TRH, suggesting that the pituitary is the primary site for both TRH and thyroid hormone influencing the secretion of TSH (Snyder and Utiger, 1972). In addition, pituitary TRH receptor number is decreased by thyroid hormone (Hinkle and Goh, 1982), and also down-regulated by prolonged exposure to TRH (Hinkle and Tashjian, 1975; Gershengorn, 1978). The regulation of TRH receptors may be of physiological significance since a decrease in the response to TRH was also noted (Gershengorn, 1978; Hinkle and Goh, 1982). Furthermore, thyroid status regulates the biosynthesis of TRH in hypothalamus as an additional feedback mechanism (Segerson, et al., 1987).

The availability of TRH to the pituitary is also under the control of its inactivating enzymes, pituitary pyroglutamyl peptidase II, and serum thyroliberinase (Bauer, 1987; Friedman and Wilk, 1985). Pituitary pyroglutamyl peptidase II and serum thyroliberinase activities are increased by thyroid hormone (White, et al., 1976; Bauer, 1987). TRH itself can down regulate pyroglutamyl peptidase II activity (Vargas, et al., 1994), suggesting that the

functional aspects of the degrading enzyme contribute to the sensitive and strict control of the TRH system. In the pituitary, pyroglutamyl peptidase II is preferentially located on lactotrope cells (Bauer, et al., 1990), suggesting that the differential localization of the inactivating enzyme and the receptor may constitute a subtle and precise regulatory system for TRH action on TSH and prolactin secretion.

1.2. TRH as a transmitter/modulator

In extrahypothalamic areas, TRH functions as a neurotransmitter or neuromodulator (Prasad, 1985). Soon after the discovery of TRH and the development of a TRH-recognizing antibody, TRH was found ubiquitously but unevenly distributed in the central nervous system. Although the highest concentration of TRH is found in the hypothalamus, about 70-80% of the total immunoactive TRH is present in extrahypothalamic areas, such as brain stem, midbrain, cerebral cortex, basal ganglia, and spinal cord (Winokur and Utiger, 1974). Immunocytochemical studies have revealed that the TRH-secreting neurons are located mostly in the medial portions of the paraventricular nuclei (Brownstein, et al., 1974) and their axonal endings are in the medial portion of the external layer of the median eminence (Lechan and Jackson, 1982). In addition to the hypothalamus, TRH-containing fibers are found in many areas of the brain, including motor nuclei of the trigeminal, facial, and hypoglossal nerves, the nucleus accumbens, the lateral septal nuclei, the stria terminalis (Hokfelt, et al., 1975a; Johansson, and Hokfelt., 1980) and anterior horn of the spinal cord (Hokfelt, et al., 1975b). Subcellular fractionation studies indicated that TRH- containing particles were of synaptosomal origin in both hypothalamic and extrahypothalamic areas (Winokur, et al., 1977).

TRH receptors are present ubiquitously but unevenly throughout the brain (Taylor and Burt, 1982) and spinal cord (Sharif and Burt, 1983a). Autoradiography provided a more precise density distribution comparison and confirmed the findings of binding studies (Palacios, 1983; Sharif, et al., 1983; Sharif, 1989). They revealed that the highest density of TRH receptors in brain was in limbic structures, especially in amygdala and hypothalamus, while lower densities were found in brain stem and cerebellum. In spinal cord, the highest level of TRH receptors was found in the dorsal and ventral gray matter. Binding experiments showed a close similarity between the TRH receptors from the the pituitary gland, retina and the other areas of CNS (Burt and Snyder, 1975; Burt, 1979; Burt and Taylor, 1980; Sharif and Burt, 1983b).

Electrophysiological effects of TRH have been documented *in vivo* and in cultured cells. Early electrophysiological studies showed that TRH inhibited neuronal excitability in hypothalamus, cerebral cortex, and cerebellum (Renaud and Martin, 1975; Winokur and Beckman, 1978). TRH inhibited Na⁺ channel activity in septal neurons (Lopez-Barneo, et al., 1990) and opened potassium currents in hippocampal CA-1 pyramidal neurons (Ebihara and Akaike, 1993). TRH had excitatory effects on rat hypoglossal motoneurons (Bayliss, et al., 1992) and dorsal motor nucleus of the vagus motoneurons (Travagli, et al., 1992) by a mechanism involving the activation of a G protein (Bayliss, et al., 1994). TRH and analogs augmented long term potentiation in the mossy fibers in guinea pig hippocampal slices, which may account for the beneficial effects of TRH on experimental memory dysfunctions (Ishihara, et al., 1992). TRH has been shown to modulate the excitatory effects of acetylcholine (Winokur and Beckman, 1978) and L-glutamate (Stocca and Nistri, 1994). In rat CA-1

neurons, TRH modulated the effect of 5-hydroxytryptamine on potassium currents (Ballerini, et al., 1994). In spinal cord, TRH induced facilitation of spinal neurotransmission (Cooper and Boyer, 1978; White, et al., 1989), which may involve NMDA receptor activation (Chizh and Headley, 1994).

If TRH acts as a transmitter, a mechanism must exist for its inactivation. Proteolytic enzymes terminate or transform the biological actions of neuropeptides. The bioavailability of neuronally released peptides is predominantly controlled by peptide degrading enzymes on synaptosomal membranes (Molineaux and Wilk, 1991). It is generally agreed that neuronally released TRH is inactivated by a highly specific and efficient synaptosomal ectoenzyme, pyroglutamyl peptidase II (Wilk, 1986). The degradation product histidylprolylamide is generally considered inactive and can be further degraded by dipeptidyl peptidase IV.

1.3. Pharmacological effects of TRH

The administration of TRH or its analogs elicits a number of various centrally mediated behavioral or pharmacological effects in man and animals. In addition to general arousal effects, analeptic, hyperthermic, cardiovascular and respiratory stimulating effects have been observed (Review in Horita, et al., 1985). Recently the neurotropic effect of TRH has attracted considerable interest. TRH was reported to enhance the neurological recovery following spinal cord impact injury, reduce spinal cord necrosis caused by injection of a substance P antagonist, and decrease the edema after spinal cord trauma in cats and rats (Faden and Salzman, 1992). A study of the structure-activity relationship of TRH analogs revealed that the beneficial effects in CNS trauma

require the integrity of the C-terminus of the tripeptide. Treatment with a TRH analog improved the outcome of brain and spinal traumatic injury in rats, in a dose-dependent manner (Faden, 1989). Thus, TRH was proposed as a pharmacological strategy for CNS trauma (Faden and Salzman, 1992). In murine ventral horn neuronal cultures, TRH was observed to have a trophic effect (Banda, et al., 1989). This action, along with its facilitation of transmission of motor neurons, has led to a clinical interest in TRH for the treatment of amyotrophic lateral sclerosis (Munsat, et al., 1989). YM-14673, a potent analog of TRH reduced the fall index in all types of ataxic mutant mice and was suggested to have beneficial effects in patients with spinocerebellar degeneration (Matsui, et al., 1994).

2. Enzymology of TRH metabolism

2.1. Metabolic pathways of TRH

Three enzymes, pyroglutamyl peptidase I, pyroglutamyl peptidase II and prolyl endopeptidase, can degrade TRH, and represent two distinct biochemical metabolic pathways (Molineaux and Wilk, 1991) (fig.1).

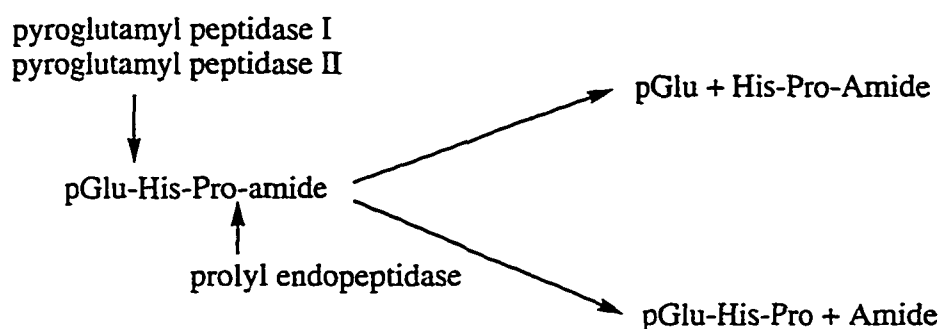


Figure 1. Metabolic pathways of TRH.

In the pyroglutamyl peptidase pathway, N-terminal pyroglutamic acid is cleaved from TRH to yield histidyl-prolineamide, which is generally considered an inactive product (Review in Prasad, 1985) although some evidence suggests it possesses a regulatory function (Peterkofsky, et al., 1983). Deamidation of TRH by prolyl endopeptidase leads to the formation of acid TRH, which is probably further metabolized to the individual amino acids by a combination of carboxypeptidase(s) and pyroglutamyl peptidase(s) (Review in Prasad, 1985).

2.2. Properties of prolyl endopeptidase

Prolyl endopeptidase is almost evenly distributed throughout the rat and mouse brain and resides in the soluble fraction (Orlowski, et al., 1979; Dresdner, et al., 1982), although a recent report presents evidence for a synaptosomal membrane-bound form of this enzyme (O'Leary and O'Connor, 1995). This enzyme exhibits a broad substrate requirement. It cleaves peptides that contain a structure of Y-Pro-X (where Y=peptide and X=peptide, amide, or aminoacylamide), including among others GnRH, substance P, angiotensin II, bradykinin, and neurotensin in addition to TRH (Orlowski, et al., 1979; Browne

and O'Cuinn, 1983). Prolyl endopeptidase has a molecular mass of 80,751 daltons (Rennex et al., 1991), a K_m of 410 mM for TRH, and an optimal pH between 7.4 and 7.6. Although the enzyme is inhibited by both cysteine and serine proteinase inhibitors, biochemical studies have established it as a serine proteinase (Review in Wilk, 1983). This has been confirmed by site-directed mutagenesis of the cloned enzyme (Rennex et al., 1991). Prolyl endopeptidase is now recognized to be a member of a newly defined sub-family of serine proteinases (McDonald and Barrett, 1986). A specific inhibitor, benzyloxycarbonyl(CBZ)-Pro-Prolinal was designed and synthesized (Wilk and Orlowski, 1983; Friedman, et al., 1984). Since then many other potent inhibitors have been synthesized.

2.3. Properties of Pyroglutamyl peptidase I

Pyroglutamyl peptidase I is also a widely distributed cytosolic enzyme (Bauer and Kleinkauf, 1980; Browne and O'Cuinn, 1983). It shows broad substrate specificity, cleaving all pyroglutamyl amino acid bonds except the pGlu-Pro bond. The substrates cleaved by this peptidase include GnRH, neurotensin and bombesin but not eledoisin which has a pGlu-Pro bond (Browne and O'Cuinn, 1983). This enzyme has an apparent mass of 24 000 daltons. The activity of soluble pyroglutamyl peptidase I is highest in the kidney and liver, with lowest activity in brain. Its distribution in the brain is quite uniform (Friedman, et al., 1986). Pyroglutamyl peptidase I is a cysteine proteinase which is highly sensitive to sulfhydryl reagents and heavy metal ions (Browne and O'Cuinn, 1983). An active site-directed reversible inhibitor 5-oxoprolinal and an active site-directed irreversible inhibitor pyroglutamyl diazomethyl ketone were developed. (Friedman, et al., 1985; Wilk, et al., 1985). Pyroglutamyl

peptidase I has been highly purified from human cerebral cortex (Lauffart et al., 1988) and cloned from bacteria (Awade, et al., 1992; Chevallier, et al., 1992).

2.4. Functional aspects of TRH degrading enzymes

Because of their cytosolic location and broad substrate requirements, prolyl endopeptidase and pyroglutamyl peptidase I are unlikely to be the enzymes that control the turnover of neuronally released TRH (Wilk, 1986). By use of specific inhibitors of these two enzymes, their role in the recovery of neuronally released TRH was evaluated. The inhibitor of each enzyme separately or in combination could not increase the recovery of TRH released from hypothalamic brain slices (Charli, et al., 1987; Mendez, et al., 1990). In the presence of benzyloxycarbonyl(CBZ)-Pro-Prolinal and pyroglutamyl diazomethyl ketone, a particulate pyroglutamyl peptidase activity was delineated (Friedman and Wilk, 1986). This enzyme was designated as pyroglutamyl peptidase II and may be the first characterized neuropeptide specific peptidase (Wilk, 1986). The inhibition of this enzyme significantly increased the recovery of neuronally released TRH (Charli, et al., 1989). It likely represents the controlling mechanism for termination of the action of neuronally released TRH (Molineaux and Wilk, 1991). Serum pyroglutamyl peptidase II (thyroliberinase) may play a role in the general control of the availability of TRH to pituitary and other peripheral organs.

3. Pyroglutamyl peptidase II

3.1. Serum thyroliberinase

Pyroglutamyl peptidase II activity was first characterized in rat serum (Taylor and Dixon, 1978). TRH was degraded to pyroglutamic acid and histidylprolineamide by rat serum. The enzyme was partially purified by conventional column chromatography. A pH optimum between 6.5 and 8.0 and a K_m of 50 mM were reported. An apparent molecular weight of 260 000 was estimated by gel filtration experiments. This serum enzyme activity was inhibited by EDTA and dithiothreitol and other metal chelating agents. These properties clearly differentiate it from pyroglutamyl peptidase I (Taylor and Dixon, 1978; Bauer and Nowak, 1979). High substrate specificity was shown by its failure to cleave the pGlu-His bond of GnRH, or the pGlu-Leu bond of neurotensin (Bauer, et al., 1981). Because of its unique specificity, it was named "thyroliberinase" (Bauer, et al., 1981). Using specific inhibitors of prolyl endopeptidase and pyroglutamyl peptidase I Friedman and Wilk (1985) demonstrated that prolyl endopeptidase and pyroglutamyl peptidase I do not contribute significantly to TRH degradation in rat serum, suggesting that this process was predominantly catalyzed by "thyroliberinase". There is no direct evidence as to the source of serum thyroliberinase.

3.2. Pyroglutamyl peptidase II in CNS

TRH-degrading activity in brain was later characterized to be present in both cytosolic and particulate fractions (Browne et al., 1981; Browne and O'Cuinn, 1983). Two forms of pyroglutamyl peptidase, one in the soluble fraction, and the other in the particulate fraction are present in rat brain (Browne, et al., 1981; O'Connor and O'Cuinn, 1984). A particulate form of pyroglutamyl peptidase was also reported in rat adenohypophysis (Bauer, 1987). The presence of particulate pyroglutamyl peptidase in the brain was further clearly

delineated by using the two specific inhibitors for prolyl endopeptidase and pyroglutamyl peptidase I. It is predominantly a brain enzyme, with highest activity in cortex and hippocampus (Friedman and Wilk, 1986). This particulate enzyme was designated as pyroglutamyl peptidase II to distinguish it from the cytosolic pyroglutamyl peptidase I (McDonald and Barrett, 1986).

Biochemical characterization of pyroglutamyl peptidase II revealed properties similar to serum thyroliberinase and these enzymes are considered to be identical (Wilk, 1986). Brain pyroglutamyl peptidase II showed a narrow substrate specificity and a large molecular mass of 230 000 daltons. The enzyme activity was inhibited by metal chelating agents such as EDTA and 1,10-phenanthroline (O'Connor and O'Cuinn, 1984). The high substrate specificity of pyroglutamyl peptidase II was confirmed with natural and synthetic substrates (Wilk and Wilk, 1989). Pyroglutamyl peptidase II does not cleave the pGlu-His bond of gonadotropin-releasing hormone, the pGlu-Leu bond of neurotensin, or a variety of other synthetic pGlu-X substrates (Wilk and Wilk, 1989). TRH is the only known naturally occurring substrate in the brain. Pyroglutamyl peptidase II was identified as a metalloproteinase and classified as a member of the zinc-dependent metalloproteinase family (Czekay and Bauer, 1993).

Pyroglutamyl peptidase II activity is located on synaptosomal and adenohipophyseal membranes with its active site oriented toward the extracellular space and thus represents a true ectoenzyme (Torres et al., 1986; Charli, et al., 1988; Cruz, et al., 1991). Inhibition of pyroglutamyl peptidase II resulted in an increased recovery of released TRH from brain slices (Charli et

al., 1989). These observations strongly suggest that pyroglutamyl peptidase II plays a critical role in the termination of the action of released TRH.

Several studies on the regional distribution of pyroglutamyl peptidase II activity in brain have been conducted. Pyroglutamyl peptidase II activity was detected in various mammalian brains, including rabbit, mouse, rat, guinea pig, hamster and pig (Vargas, et al., 1992). The distribution of pyroglutamyl peptidase II activity in brain is not homogeneous. The highest activity in rat brain is found in olfactory bulb and hippocampus, followed by cerebral cortex and hypothalamus. Brainstem and the cervical part of the spinal cord have lowest activities (Vargas, et al., 1987). In rabbit brain, the distribution pattern is similar to rat. The spinal cord of the rabbit contains significant but low amounts of pyroglutamyl peptidase II activity, with a relatively homogeneous distribution throughout the cervical, thoracic and lumbar sections. Although pyroglutamyl peptidase II is predominantly found in brain, some significant activity is found in some peripheral organs such as lung, testes, spleen and prostate (Vargas, et al., 1992). It was reported that pyroglutamyl peptidase II activity is present in rabbit retinal membranes with the amount exceeding any of the non-CNS tissues thus far studied (Wilk, et al., 1988). Relatively high enzymatic activity is associated with cultured neuronal cells from embryonic rat brain while glial cells are virtually devoid of this activity. Lower but significant activity is found in anterior pituitary cells and activity is correlated with the distribution of lactotrophic cells. It has been proposed that pyroglutamyl peptidase II is specifically localized on prolactin secreting cells (Bauer, et al., 1990). It was suggested that pyroglutamyl peptidase II is not localized on TRH neurons but probably on the target cell. This proposal was based on the observation that no change in pyroglutamyl peptidase II was detected following chemical

degeneration of serotonin-TRH cells that project from raphe nuclei to the spinal cord (Joseph-Bravo, et al., 1994).

3.3. Regulation of pyroglutamyl peptidase II

Pyroglutamyl peptidase II is subject to various types of regulation. The TRH-degrading activity in rat serum is significantly reduced by thiouracil-induced hypothyroidism and increased by triiodothyronine (White, et al., 1976). Adenohypophyseal pyroglutamyl peptidase II is increased by triiodothyronine. This effect has a rapid onset and is dose-dependent. Likewise, propylthiouracil produces hypothyroidism and down-regulates pituitary pyroglutamyl peptidase II activity (Bauer, 1987). Estrogen suppresses pyroglutamyl peptidase II activity in rat anterior pituitary and also suppresses the stimulating effect of triiodothyronine (Bauer, 1988). Acute application of triiodothyronine increases pyroglutamyl peptidase II activity in rat frontal cortex and pituitary, while chronic treatment with triiodothyronine elevates its activity in frontal cortex and serum (Suen and Wilk, 1989). Administration of thyroid hormone increases the rat serum TRH degrading activity (White, et al., 1976; Emerson and Wu, 1987) which by the application of inhibitors of prolyl endopeptidase and pyroglutamyl peptidase I is attributed to pyroglutamyl peptidase II (thyroliberinase) (Friedman and Wilk, 1985). Thyroid status also regulates the liver particulate pyroglutamyl peptidase II (Scharfmann, et al., 1990). In humans, it was observed that serum pyroglutamyl peptidase II activity is influenced by body weight and thyroid status. This phenomenon indicates that serum pyroglutamyl peptidase II activity is regulated by, or regulates, metabolic pathways (Friedman, et al., 1995). These data suggest a role for pyroglutamyl peptidase II in controlling responses of pituitary and brain to TRH.

The regulation of pyroglutamyl peptidase II by TRH was demonstrated in primary cultures of adenohypophyseal cells. Both TRH and phorbol ester were shown to diminish the activity of pyroglutamyl peptidase II and their maximal effects were not additive. This result suggested that TRH regulation of pyroglutamyl peptidase II activity may contribute to the pituitary response to TRH (Vargas, et al., 1994). In Y-79 human retinoblastoma cells, pyroglutamyl peptidase II is rapidly inactivated by treatment with phorbol ester. These cells contain relatively high amounts of pyroglutamyl peptidase II but lack TRH receptors. Since TRH receptor activation is coupled to an activation of protein kinase C, the effect of phorbol ester supported the possibility that TRH could regulate pyroglutamyl peptidase II activity (Suen and Wilk, 1990). Further experiments showed that the inactivation was a biphasic process. The rapid onset phase was due to pyroglutamyl peptidase II phosphorylation by protein kinase C. The later slow and persistent phase was the result of suppression of synthesis of pyroglutamyl peptidase II (Suen and Wilk, 1991).

3.4. cDNA cloning of pyroglutamyl peptidase II

A cDNA encoding pyroglutamyl peptidase II has been cloned (Schauder, et al., 1994). It is a cDNA encoding a protein of 1025 amino acids. Transfection of this cDNA in COS-7 cells resulted in expression of an active ectoenzyme displaying the characteristics of the TRH degrading enzyme. Hydrophobicity analysis predicts a transmembrane-spanning domain near the amino terminal. The amino terminal is presumably an intracellular part of the enzyme, containing a threonine residue which might be a phosphorylation site for protein kinase C. The large extracellular domain contains the HEXXH zinc-

binding motif at the position of 441-445 with a second glutamic acid separated by 18 amino acids. This demonstrates that this enzyme belongs to the zinc-dependent metalloproteinase family. Also this domain contains a consensus sequence for tyrosine sulfation and 12 putative N-glycosylation sites. The cloning and expression of pyroglutamyl peptidase II opens the opportunity for further exploration of the structure, function and regulation of this enzyme at the molecular level.

4. Aims of Our Studies

The prior efforts made toward the purification of pyroglutamyl peptidase II both in this laboratory and elsewhere were unable to achieve homogeneity on SDS-PAGE. This had hampered the understanding of the structure of pyroglutamyl peptidase II and prevented the development of antibodies (Wilk and Wilk, 1989; O'Connor and O'Cuinn, 1984, 1985). Difficulties encountered were due to the low abundance and instability of the enzyme. Several attempts in this laboratory to sequence a tryptic peptide from the purified enzyme yielded a sequence with 100% homology to creatine phosphokinase (CPK) (Wilk, unpublished data), indicating that CPK was a major contaminant in the preparations. Our initial aim therefore, was to successfully purify pyroglutamyl peptidase II free of CPK and to obtain the amino acid sequence of tryptic peptides for subsequent cDNA cloning. The purified enzyme was also to be used to prepare antibodies. While these studies were in progress and a successful purification scheme yielding the sequence of two tryptic peptides had been developed, the cloning of the cDNA was published (Schauder et al., 1994).

When the cDNA information was published, we turned our attention to the quantitation of the regional distribution of the mRNA of pyroglutamyl peptidase II in rats and its regulation. In the literature, there was no information about the brain regional distribution of its mRNA level, and the information about the organ distribution was very limited (Schauder, et al., 1994). As pyroglutamyl peptidase II activities in pituitary, frontal cortex, serum, and liver were up-regulated by thyroid hormone (White, et al., 1976; Emerson and Wu, 1987; Bauer, 1987; Suen and Wilk, 1989; Scharfmann, et al., 1990), we sought to examine the potential change of mRNA levels of pyroglutamyl peptidase II, under the same mode of treatment with thyroid hormone as used in studies measuring enzymatic activity.

TRH was proposed to possess therapeutic value for CNS trauma and degenerative diseases (Faden and Salzman, 1992; Munsat, et al., 1989; Matsui, et al., 1994), and has been used clinically for diagnosis and treatment. Specific receptors mediate the action of TRH which is a G protein coupled receptor linked to the phospholipase C pathway (Review in Gershengorn, 1993). We were interested in studying the potential regulatory effects of TRH on pyroglutamyl peptidase II in vivo and in cell culture systems.

The GH3 cell is a well characterized prolactin and growth hormone secreting rat pituitary tumor cell line. It contains functional TRH receptors (Aragay, et al., 1992)) as well as low but measurable levels of pyroglutamyl peptidase II (Wilk, et al., 1988)). We were interested in establishing the GH3 cell as a model system for studying pyroglutamyl peptidase II mRNA regulation by thyroid hormone and TRH, and in dissecting the mechanism of any changes.

Epidermal growth factor (EGF) has been shown to remarkably increase the population of prolactin-secreting cell types in GH3 cultures (Felix, et al., 1995). As pyroglutamyl peptidase II is proposed to be preferentially located on lactotrope cells in the pituitary (Bauer, et al., 1990), the shift to a prolactin secreting cell population may result in an increase in this enzyme. Therefore, we wished to extend our studies to the effects of EGF on pyroglutamyl peptidase II mRNA in GH3 cultures.

Chapter 2

Purification and Characterization of Subunit Structure of Pyroglutamyl Peptidase II

Abstract

Pyroglutamyl peptidase II was purified 10,000 fold from rabbit brain to apparent homogeneity as judged by SDS-PAGE and non dissociating gel electrophoresis. The subunit structure of rabbit brain pyroglutamyl peptidase II was established as a homodimer with a subunit molecular mass of 115 kDa. We optimized a purification procedure consisting of trypsinization of washed brain membranes, Q-Sepharose ion exchange chromatography, Sephadex G 300 gel filtration, lentil-lectin Sepharose chromatography and phenyl Sepharose hydrophobic interaction chromatography. The specific enzymatic activity was estimated as 135 μ moles pGlu-His-Pro-2-naphthylamide degraded/mg/h. The properties of pyroglutamyl peptidase II are consistent with that of a membrane glycosylated protein. The amino acid sequences of two tryptic peptides were obtained from the purified enzyme. Immunization of mice with the purified enzyme resulted in an antiserum recognizing rabbit brain pyroglutamyl peptidase II.

Thyrotropin-releasing hormone (TRH) functions as a hypothalamic hypophyseal hormone, neurotransmitter or neuromodulator in the central nervous system (Review, Prasad, 1985). As a synaptosomal ectoenzyme with high substrate specificity, pyroglutamyl peptidase II was proposed to be the enzyme controlling the turnover of neuronally released TRH (Review, Wilk, 1986). This was further supported by the observation that the inhibition of pyroglutamyl peptidase II resulted in an increase in the recovery of TRH released from brain slices by K^+ depolarization (Charli, et al, 1989). The presence of pyroglutamyl peptidase II in serum (thyroliberinase) and pituitary may contribute to the availability of TRH to pituitary and other organs. Pyroglutamyl peptidase II activities in pituitary, frontal cortex, serum and liver are regulated by thyroid hormone (White, et al, 1976; Bauer, 1987; Scharfmann, et al, 1990; Suen and Wilk, 1989), suggesting that the functional status of the degrading enzyme contributes to the precise and sensitive control of the action of TRH.

We and others have pursued the purification of pyroglutamyl peptidase II from rabbit and guinea pig brain (Wilk and Wilk, 1989; O'Connor and O'Cuinn, 1984, 1985), but have been unable to achieve homogeneity on SDS-PAGE until recently. The difficulty was due to the low abundance and instability of the enzyme. This prevented the determination of the specific activity of the pure enzyme, its subunit structure and the development of specific antibodies. Cloning of the cDNA encoding this enzyme was also delayed.

We now report a optimized purification procedure by which pyroglutamyl peptidase II can be purified from rabbit brain to apparent homogeneity as judged by both SDS-PAGE and nondissociating gel electrophoresis. The

subunit structure of pyroglutamyl peptidase II was characterized to be a homodimer. The biochemical properties of the enzyme revealed after purification were consistent with that of a glycosylated membrane protein. We were also able to obtain two amino acid sequences of tryptic peptides of the pure enzyme. Immunization of mice with the purified enzyme resulted in an antiserum recognizing rabbit brain pyroglutamyl peptidase II.

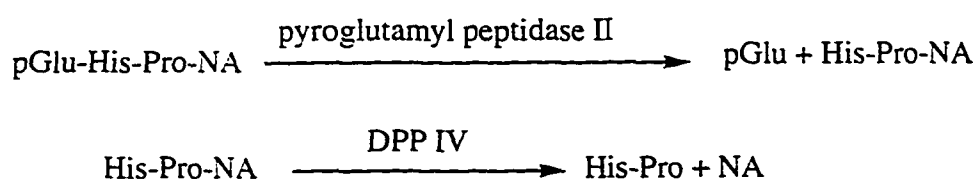
Materials and Methods

Materials

Rabbit brain (young, unstripped) was from Pel Freez, Rogers, AK. The substrate pGlu-His-Pro-NA was synthesized as previously described (Wilk and Wilk, 1989). DPP IV was purified from rat kidney as described (Yoshimoto and Walter, 1977). Trypsin, trypsin inhibitor, malonic acid, sodium azide, α -methyl-D-glucoside, glycerol, SDS, Tween-20, and Trizma base were purchased from Sigma. Q-Sepharose, Sephadex G-300, lentil lectin Sepharose, and Phenyl Sepharose were from Pharmacia.

Measurement of pyroglutamyl peptidase II enzymatic activity

The method for determination of enzymatic activity was previously developed in this laboratory (Wilk and Wilk, 1989). Enzymatic activity is determined with the synthetic substrate pGlu-His-Pro-naphthylamide (NA) in a coupled assay in the presence of an excess amount of dipeptidyl peptidase IV (DPP IV):



The reaction mixture consists of 50 mM sodium phosphate buffer (pH 7.5), 5 μ l of DPP IV (1 μ g protein) and 5 μ l of substrate (10 mM in DMSO), and pyroglutamyl peptidase II, in a total volume of 125 μ l. Tubes are incubated at 37⁰ C for 30 min to 2 h, and the reaction terminated by addition of 125 μ l of 10% TCA. Free NA is quantitated by a colorimetric reaction (Goldbarg and

Rutenburg, 1958) and absorbance of the chromogen is read at 580 nm. One unit of the enzyme is defined as the amount of enzyme releasing 1 $\mu\text{mol/h}$.

Measurement of creatine phosphokinase (CPK) activity

Measurement of CPK activity was performed with a Sigma Diagnostics Kit. Briefly, 3 ml of H_2O was added to one vial of kit reagent (Cat. No. 45-1), and the vial mixed well. One ml of this solution was added to a spectrophotometric cuvette together with 1 - 10 μl of sample to be assayed. The absorbance at 340 nm was recorded at 5 min and 10 min exactly. One unit of CPK was defined as the change of 0.1 absorbance unit at 340 nm/ per min incubation.

Solubilization of the membrane-bound pyroglutamyl peptidase II

Rabbit brain (150 grams) was homogenized in 20 mM sodium phosphate buffer (pH 7.5), and centrifuged at 100 000 g for 45 minutes. The resulting pellet was washed 5 times with the same buffer and recovered after centrifugation. The washed pellets were resuspended with phosphate buffer, and detergents or trypsin were added at the desired concentration. Solubilization was carried out at the indicated temperature and incubation time, and was followed by ultracentrifugation. The supernatant representing the solubilized membrane fraction was subjected to column chromatography.

Enzyme purification

The procedure developed was a modification and optimization of methods of Wilk and Wilk, 1989 and Czech, et al., 1994. The washed pellets of rabbit brain were subjected to trypsinization at various concentrations of trypsin and various times and temperatures of incubation. Trypsinization was terminated by the same concentration of trypsin inhibitor. After centrifugation at

100 000 g for 1 h, the supernatant was applied to a 5 cm X 20 cm Q-Sepharose column. The column was then washed with 20 mM sodium phosphate buffer, pH 7.5, until the absorbance at 280 nm of the outflow was 0.1 or less. Elution of the enzyme was achieved by a linear gradient of 0 - 0.6 M sodium chloride in the same buffer. Fractions with enzymatic activity were collected and concentrated to 10 ml in an ultrafiltration cell under positive nitrogen pressure. The concentrated sample was subjected to gel filtration on a Sephadex G-300 column equilibrated with 0.1 M sodium chloride in 20 mM sodium phosphate buffer (pH 7.5). The active fraction was chromatographed on a lentil lectin Sepharose column (1 cm X 17 cm) which was equilibrated with 0.01 M malonic acid/0.3 M sodium chloride/6 mM sodium azide (pH 7.3). The column was washed with at least 8 volumes of the buffer and eluted with 0.5 M α -methyl-D-glucoside in the same buffer. The final purification step was completed by hydrophobic interaction chromatography on a phenyl Sepharose column. After the application of the sample, the column was washed with 30 ml of 50 mM sodium phosphate/150 mM sodium chloride (pH 7.5), then 30 ml of 50 mM sodium phosphate/200 mM sodium chloride/30% glycerol. The enzyme was eluted with 50 mM sodium phosphate/50 mM sodium chloride/60% glycerol. The eluate was collected in one ml fractions at a rate of 12 ml/hr. In the earlier studies, acid precipitation was carried out by addition of 1 M acetic acid to pH 5.0. When DEAE affi gel blue column chromatography (2.3 X 10 cm) was used, the active fraction of enzyme was applied, the column was washed with 100 ml sodium phosphate buffer (10 mM, pH 7.5), then eluted with 0-20 mM ATP in the same buffer, followed by elution with 0.1-0.5 M NaCl in this buffer.

Gel electrophoresis

Purified pyroglutamyl peptidase II was electrophoresed on 5% nondissociating polyacrylamide gels in a phosphate buffer system (pH 7.5) as described (Wilk and Wilk, 1989). SDS/PAGE was performed on 9% gels (Laemmli, 1970). The gels were stained with Coomassie blue or silver.

Immunoblotting:

Samples were separated on a 9% SDS-PAGE minigel run at 250-300V for 30-45 min. The proteins were transferred to an Immobilon membrane (Millipore, Bedford, MA) in a transfer apparatus containing transfer buffer of 25 mM Tris base, 192 mM glycine, and 15% methanol at 70 V for 1 hr. After transfer, the membrane was washed for 10 min. in TBS (Tris-buffered saline) and preincubated with 5% BSA in TBS for 1 hr. The membrane was then washed 2 times with TTBS (0.5% tween 20 in TBS), for 5 min each wash. The primary antibody at satisfactory dilution in TTBS containing 1% BSA was incubated for at least 2 hr, and in most cases, overnight. The unbound antibody was removed with 2 washes of TTBS of 5 min each. The membrane was incubated with goat anti-rabbit serum at 1:3000 dilution in TTBS/BSA for 1 hr. This was followed by 2 washes of TTBS to remove unbound antibody, and one wash of TBS to remove tween-20. Color development was performed by incubating with a TMB membrane Peroxidase Substrate System (vectastain ABC kit, Vector Laboratory) for 2-10 min.

Amino acid sequencing of tryptic peptides

20 µg of purified pyroglutamyl peptidase II in 500 µl buffer was sent to the Peptide Biology Laboratory, Baker Medical Research Institute in Australia for tryptic peptide sequencing. Two sequences were obtained.

Generation of antiserum

A laboratory manual was followed as a general guideline (Harlow and Lane eds., 1988). The purified protein was used to produce mouse monoclonal antibodies in the Hybridoma Core Facility. 10 - 20 µg of protein was used to immunize and boost each mouse. A total of 3 mice were used. Serum was obtained after the first and second boosts and tested for its titer against purified pyroglutamyl peptidase II. Purified protein was used as antigen in the radioimmunoassay for screening the titers of antiserum and hybridoma culture medium. The quality of antiserum and hybridoma were also examined by Western blotting against the purified protein and washed brain pellets.

Determination of Protein concentrations

Measurement of absorbance at 280 nm was used to estimate the protein concentration of the column eluates. The protein concentrations of the final eluates from phenyl Sepharose were estimated by visual comparison of silver stained SDS-PAGE gels to known concentrations of β -galactosidase. Quantitation of protein concentration in all other cases was by the method Lowry, et al., (1951).

Results

Solubilization of pyroglutamyl peptidase II

We have attempted to solubilize pyroglutamyl peptidase II by several common detergents. CHAPS, Triton X-100, Lubrol, NP80, and deoxycholate can reasonably solubilize significant amounts of pyroglutamyl peptidase II activity (figs. 1- 5), however, all of these detergents destabilized the enzyme (Table 1). Pyroglutamyl peptidase II activity remained stable following limited proteolysis of membranes with trypsin. We observed that different trypsinization conditions affected the structural integrity of the enzyme. Strong trypsinization, (trypsin 80 $\mu\text{g/ml}$, 37 $^{\circ}\text{C}$ for 1 hr; Wilk and Wilk, 1989) resulted in a predominant band of 97 kDa on SDS-PAGE, and a second band of 110 kDa, although enzymatic activity was preserved and homogeneity on native gel electrophoresis was achieved (data not showed). Table 2 summarizes the purification result after strong trypsinization (trypsin 80 $\mu\text{g/ml}$, 37 $^{\circ}\text{C}$ for 1 hr; Wilk and Wilk, 1989). We optimized the trypsin final concentration to 2 $\mu\text{g/ml}$ (fig. 6) and trypsinization was carried on at 4 $^{\circ}\text{C}$ for 15 min (fig. 7). This resulted in a predominant band of 115 kDa on SDS-PAGE with greatest recovery of pyroglutamyl peptidase II activity.

Enzyme purification

(1) Pyroglutamyl peptidase II activity was firmly membrane-bound; several common buffers with high NaCl concentrations were unable to release the enzymatic activity from the particulate fraction of a brain homogenate. CPK activity co-purified with pyroglutamyl peptidase II during Sephadex G 300 (fig. 8) and Q-Sepharose chromatographies (fig. 9) as well as phenyl Sepharose chromatography (fig. 10), and was present as the dominant band on

SDS/PAGE in the final preparation. DEAE affi Gel blue (fig. 11) or lentil lectin Sepharose (fig. 12) effectively separated the CPK and pyroglutamyl peptidase II activities. The binding to a lentil lectin Sepharose column indicated that pyroglutamyl peptidase II is a glycosylated membrane protein (fig. 12).

(2) A procedure was developed by optimizing the steps of gentle trypsinization, Q-Sepharose ion-exchange chromatography (fig. 13), Sephadex G 300 gel filtration (fig. 14), lentil lectin Sepharose (fig. 15) interaction, and Phenyl Sepharose hydrophobic interaction chromatography (fig. 16). Pyroglutamyl peptidase II was purified 10000 fold by this procedure (Table 3). Apparent homogeneity on a native gel (fig. 17) and SDS-PAGE was achieved (fig. 18). The intensity of the band on a native gel correlated to the peak of pyroglutamyl peptidase II enzymatic activity (fig. 17). The monomer molecular weight was calculated as 115 kDa (fig. 19). The protein concentration of the final purified preparation was too low to be quantitated by method of Lowry, and thus was estimated by the visual comparison to known concentrations of β -galactosidase on SDS-PAGE and visualized with silver staining (fig. 20). The specific enzymatic activity of pyroglutamyl peptidase II was estimated as 135 units/mg, which well exceeds the previous reported value of 35 units/mg (Wilk and Wilk, 1989).

A single band on SDS-PAGE of 115 kDa molecular mass was obtained (fig. 18; fig. 20). This band was present throughout the purification and correlated with the enzymatic activity of pyroglutamyl peptidase II. Since the mass of the native enzyme as determined by gel filtration experiments was 230 kDa (Wilk and Wilk, 1989), a homodimeric structure was established.

Kinetic studies with pyroglutamyl peptidase II purified by strong trypsinization and facile trypsinization showed that their K_m 's were similar (facile trypsinization, 50 μ M; strong trypsinization, 65 μ M).

Several attempts by the Peptide Biology Laboratory in Australia to sequence the band on SDS-PAGE failed, possibly due to its low concentration and to low recovery from the gel. Two amino acid sequences of the tryptic peptides of pyroglutamyl peptidase II were obtained from 20 µg of pure preparation in solution (LISGVXEF; LYQDELFQXL). These two sequences were subsequently found within the recently reported cDNA sequence (amino acid 962-969, and 1009-1119 respectively Schauder, et al., 1994), confirming that it is the same entity.

Generation and testing of antiserum

One out of the three mice immunized with purified pyroglutamyl peptidase II showed a good recognition of the purified enzyme after three immunizations. The antiserum recognized one band in the rabbit brain homogenate following SDS-PAGE, which was in the same position as the purified rabbit brain enzyme (fig 21). The antiserum also recognized the purified enzyme by radioimmunoassay (fig. 22).

Discussion

Pyroglutamyl peptidase II was previously purified 5000 fold to apparent homogeneity on native polyacrylamide gels by a method already developed in this laboratory. This procedure employed Sephadex G 200 gel filtration, DEAE Sepharose ion exchange chromatography, and phenyl Sepharose hydrophobic interaction chromatography to purify pyroglutamyl peptidase II from rabbit brain. The specific enzyme activity was estimated to be 35 units/mg. However, there were three major bands on SDS-PAGE (Wilk and Wilk, 1989, and unpublished observations). The dominant band of 42 kDa was subsequently sequenced and found to be 100% homologous with creatine phosphokinase (Wilk, unpublished data). Creatine phosphokinase (CPK) activity was found to copurify and be present in the final purified preparation. Our current studies ruled out any physical or functional relationship between CPK and pyroglutamyl peptidase II.

Initial attempts to solubilize the enzyme with detergents were unsuccessful. Several common detergents such as CHAPS, lubrol, Triton X-100, NP-40, and deoxycholate failed to effectively solubilize a stable preparation of pyroglutamyl peptidase II. A transient activation of the enzyme activity was followed by rapid inactivation. However, limited trypsinization was successful in releasing significant amounts of active enzyme from washed brain synaptosomal membranes. The enzyme activity was maintained well in phosphate buffer but not in Tris buffer. The facile trypsinization of pyroglutamyl peptidase II from membranes indicates that the serum pyroglutamyl peptidase II most likely arises from limited proteolysis of the membrane enzyme.

DEAE-Affi gel Blue and lentil lectin Sepharose columns were able to remove CPK activity with good recovery of pyroglutamyl peptidase II activity. The binding of pyroglutamyl peptidase II to a lentil lectin column indicated that this enzyme is a glycosylated protein. This modified procedure resulted in a preparation free of CPK activity, and was homogeneous on native polyacrylamide gels. On SDS-PAGE, a dominant band of 97 kDa was found, and a second band of 110 kDa was also present. One to two units of the purified enzyme was run on SDS-PAGE. After staining with Coomassie brilliant blue, the band of 97 kDa was excised and sent for sequencing. However, no information was obtained because of the low concentration of the sample.

By a facile trypsinization and optimizing column chromatography, pyroglutamyl peptidase II was purified to apparent homogeneity on a native polyacrylamide gel and also on SDS-PAGE. The correlation between the enzyme activity and staining intensities of bands on SDS-PAGE leaves no doubt about the identification of the enzyme. Gentle trypsinization may contribute to the conservation of the intact enzyme structure, whereas over-trypsinization likely accounted for the more complicated gel pattern previously observed. Furthermore, the amino acid sequences of two tryptic peptides obtained are found in the cDNA sequence published later by Schauder, et al., (1994).

There has been no successful antibody for this enzyme so far. A specific antibody is important for anatomical and functional studies. The availability of pure enzyme provides a direct approach for antibody generation. We were able to obtain a mouse antiserum that recognized the purified pyroglutamyl peptidase II as demonstrated by radioimmunoassay and Western blotting.

Unfortunately, subsequent attempts to develop monoclonal antibodies failed due to technical reasons, and the very limited quantity of the antiserum obtained prevented its application to other studies.

In summary, we have successfully purified pyroglutamyl peptidase II from rabbit brain membranes and established its subunit structure. The availability of a purification procedure and pure enzyme may promote subsequent studies toward a fuller understanding of its structure and function.

Figure Legends

Figure 1. Solubilization of pyroglutamyl peptidase II (PPII) by CHAPS. A washed brain pellet was incubated with CHAPS at different concentrations at room temperature for 1 hr, and then subjected to ultracentrifugation. The enzymatic activity in the supernatant was measured at 580 nm (chromogen released from the substrate pGlu-His-Pro-NA). The protein concentration was measured by the Lowry method at 640 nm.

Figure 2. Solubilization of pyroglutamyl peptidase II (PPII) by Triton X-100. A washed brain pellet was incubated with Triton X-100 at different concentrations at room temperature for 1 hr, and then subjected to ultracentrifugation. The enzymatic activity in the supernatant was measured at 580 nm (chromogen released from the substrate pGlu-His-Pro-NA). The protein concentration was measured by the Lowry method at 640 nm.

Figure 3. Solubilization of pyroglutamyl peptidase II (PPII) by deoxycholate. A washed brain pellet was incubated with deoxycholate at different concentrations at room temperature for 1 hr, and then subjected to ultracentrifugation. The enzymatic activity in the supernatant was measured at 580 nm (chromogen released from the substrate pGlu-His-Pro-NA). The protein concentration was measured by the Lowry method at 640 nm.

Figure 4. Solubilization of pyroglutamyl peptidase II (PPII) by NP-80. A washed brain pellet was incubated with NP-80 at different concentrations at room temperature for 1 hr, and then subjected to ultracentrifugation. The enzymatic activity in the supernatant was measured at 580 nm (chromogen released from

the substrate pGlu-His-Pro-NA). The protein concentration was measured by the Lowry method at 640 nm.

Figure 5. Solubilization of pyroglutamyl peptidase II (PPII) by Lubrol-px. A washed brain pellet was incubated with Lubrol-px at different concentrations at room temperature for 1 hr, and then subjected to ultracentrifugation. The enzymatic activity in the supernatant was measured at 580 nm (chromogen released from the substrate pGlu-His-Pro-NA). The protein concentration was measured by the Lowry method at 640 nm.

Figure 6. Dose-effect of trypsinization of pyroglutamyl peptidase II. A washed brain pellet was incubated with trypsin at different concentrations at 4 °C for 30 min, and then subjected to ultracentrifugation. The enzymatic activity in the supernatant was measured at 580 nm (chromogen released from the substrate pGlu-His-Pro-NA). The protein concentration was measured by Lowry's method at 640 nm.

Figure 7. Time effect of trypsinization of pyroglutamyl peptidase II. A washed brain pellet was incubated with 2µg/ml trypsin at 4 °C from 5 min to 60 min, and then subjected to ultracentrifugation. The enzymatic activity in the supernatant was measured at 580 nm as NA released from the substrate pGlu-His-Pro-NA. The protein concentration was measured by Lowry's method at 640 nm.

Figure 8. Sephadex G 300 chromatography of pyroglutamyl peptidase II following strong trypsinization (80 µg/ml trypsin at 37 °C for 1 hr) and acid precipitation. Enzymatic activity was determined in fractions by measuring the absorbance at 580 nm of the chromogen formed from liberated NA as described

in Materials and Methods. Protein concentration was estimated in the fractions by the absorbance at 280 nm. Creatine phosphokinase activity was measured by determining the change of absorbance at 340 nm as described in Materials and Methods.

Figure 9. Q-Sepharose chromatography of pyroglutamyl peptidase II following strong trypsinization (80 μ g/ml trypsin at 37 °C for 1 hr), acid precipitation, and Sephadex G 300 chromatography. Enzymatic activity was determined in fractions by measuring the absorbance at 580 nm of the chromogen formed from liberated NA as described in Materials and Methods. Protein concentration was estimated in the fractions by the absorbance at 280 nm. Creatine phosphokinase activity was measured by determining the change in absorbance at 340 nm as described in Materials and Methods.

Figure 10. Phenyl Sepharose chromatography of pyroglutamyl peptidase II following strong trypsinization (80 μ g/ml trypsin at 37 °C for 1 hr), acid precipitation, Sephadex G300 chromatography and Q-Sepharose chromatography. Enzymatic activity was determined in fractions by measuring the absorbance at 580 nm of the chromogen formed from liberated NA as described in Materials and Methods. Protein concentration was determined by Lowry's method which is shown by the absorbance at 640 nm. Creatine phosphokinase activity was measured by determining the change of absorbance at 340 nm as described in Materials and Methods.

Figure 11. DEAE affi gel blue chromatography of pyroglutamyl peptidase II followed a strong trypsinization (80 μ g/ml trypsin at 37 °C for 1 hr) acid precipitation, Sephadex G 300, and Q-Sepharose chromatography. Enzymatic

activity was determined in fractions by measuring the absorbance at 580 nm of the chromogen formed from liberated NA as described in Materials and Methods. Protein concentration was estimated in the fractions by the absorbance at 280 nm. Creatine phosphokinase activity was measured by determining the change of absorbance at 340 nm as described in Materials and Methods.

Figure 12. Lentil lectin Sepharose chromatography of pyroglutamyl peptidase II following strong trypsinization (80 μ g/ml trypsin at 37 °C for 1 hr) acid precipitation, Sephadex G 300, and Q-Sepharose chromatography. Enzymatic activity was determined in fractions by measuring the absorbance at 580 nm of the chromogen formed from liberated NA as described in Materials and Methods. Protein concentration was determined by Lowry's method which is shown by the absorbance at 640 nm. Creatine phosphokinase activity was measured by determining the change of absorbance at 340 nm as described in Materials and Methods.

Figure 13. Q-Sepharose chromatography of pyroglutamyl peptidase II following facile trypsinization (2 μ g/ml trypsin at 4 °C for 15 min). Enzymatic activity was determined in fractions by measuring the absorbance at 580 nm of the chromogen formed from liberated NA as described in Materials and Methods. Protein concentration was estimated in the fractions by the absorbance at 280 nm.

Figure 14. Sephadex G 300 chromatography of pyroglutamyl peptidase II following facile trypsinization (2 μ g/ml trypsin at 4 °C for 15 min) and Q-Sepharose chromatography. Enzymatic activity was determined in fractions by

measuring the absorbance at 580 nm of the chromogen formed from liberated NA as described in Materials and Methods. Protein concentration was estimated in the fractions by the absorbance at 280 nm.

Figure 15. Lentil lectin Sepharose chromatography of pyroglutamyl peptidase II following facile trypsinization (2 μ g/ml trypsin at 4 °C for 15 min), Q-Sepharose chromatography and Sephadex G 300 chromatography. Enzymatic activity was determined in fractions by measuring the absorbance at 580 nm of the chromogen formed from liberated NA as described in Materials and Methods. Protein concentration was determined by Lowry's method which was reflected by the absorbance at 640 nm.

Figure 16. Phenyl Sepharose chromatography of pyroglutamyl peptidase II following a facile trypsinization (2 μ g/ml trypsin at 4 °C for 15 min), Q-Sepharose chromatography and Sephadex G 300 chromatography. Enzymatic activity was determined in fractions by measuring the absorbance at 580 nm of the chromogen formed from liberated NA as described in Materials and Methods. Protein concentration was estimated by visual comparison with known concentrations of β -galactosidase after SDS/PAGE and silver staining.

Figure 17. Polyacrylamide gel electrophoresis (5% gel) of pyroglutamyl peptidase II after the final step of purification. A parallel gel was sliced into 3 mm pieces and incubated with the substrate overnight to determine the enzymatic activity. The position of the band corresponded to a narrow peak of enzyme activity.

Figure 18. SDS-PAGE (9% gel) of rabbit brain pyroglutamyl peptidase II after the final step of purification. The fraction was eluted with 60% glycerol from a Phenyl Sepharose column. The intensity of the band corresponded with enzymatic activity. The enzymatic activity was as follows: lane 1, 0.03 unit/ml; lane 2, 0.36 unit/ml; lane 3, 0.32 unit/ml; lane 4, 0.28 unit/ml; lane 5: 0.27 units/ml; lane 6, 0.12 units/ml; lane 7: 0.09 units/ml; lane 8, 0.08 unit/ml; lane 9, 0.03 unit/ml. The molecular weight markers were 116 kDa, 94 kDa, 67 kDa, 43 kDa, 20 kDa, 14.4 kDa, from the top.

Figure 19. The monomer molecular weight of pyroglutamyl peptidase II. The enzyme was purified to homogeneity on SDS-PAGE. The apparent molecular weight of pyroglutamyl peptidase II is estimated as 115 kDa.

Figure 20. Estimation of the protein concentration of pyroglutamyl peptidase II. Different concentrations of β -galactosidase were used as standards. By comparing the intensity following silver staining, the protein concentration of pyroglutamyl peptidase II was estimated as 0.04 mg/ml. The specific activity was thus established 135 unit/mg. From left to right, the amount of β -galactosidase is 0.05, 0.10, 0.20, 0.40, 0.80, 1.2, 1.6 μ g respectively. 5 μ l of pyroglutamyl peptidase II was used for the determination. All samples were brought up to a volume of 20 μ l for loading. The molecular weight markers are 116 kDa, 94 kDa, 67 kDa, 43 kDa, 20 kDa, from the top of the gel.

Figure 21. Western blot of the mouse antiserum against purified pyroglutamyl peptidase II and the corresponding band from a rabbit brain homogenate. The antiserum recognized one band of the rabbit brain homogenate on SDS-PAGE, which was in the same position as the purified rabbit brain enzyme. Lane 1:

purified rabbit brain pyroglutamyl peptidase II; Lane II: partially purified pyroglutamyl peptidase II (after Sephadex G 300 chromatography and Q-Sephadex chromatography); Lane 3: washed rabbit brain pellet; Lane 4: rat kidney homogenate.

Figure 22. Recognition by radioimmunoassay of pyroglutamyl peptidase II by a mouse antiserum generated by immunizing with purified rabbit enzyme. Purified rabbit brain pyroglutamyl peptidase II was coated on plates and reacted with PBS/BSA, normal mouse serum and mouse antiserum with 1: 1000 and 1: 10000 dilutions. Radiolabelled second antibody against mouse serum was added. Unbound antibody was washed away with PBS (pH 7.4). CPM represents the binding of antibody to antigen.

Figure 1

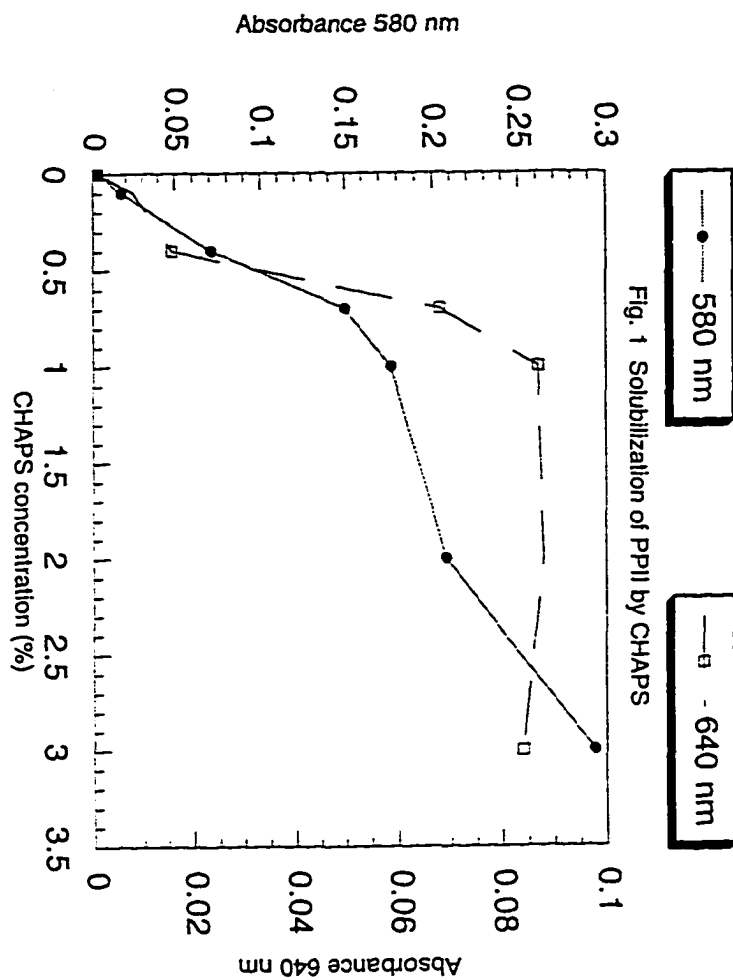


Figure 2

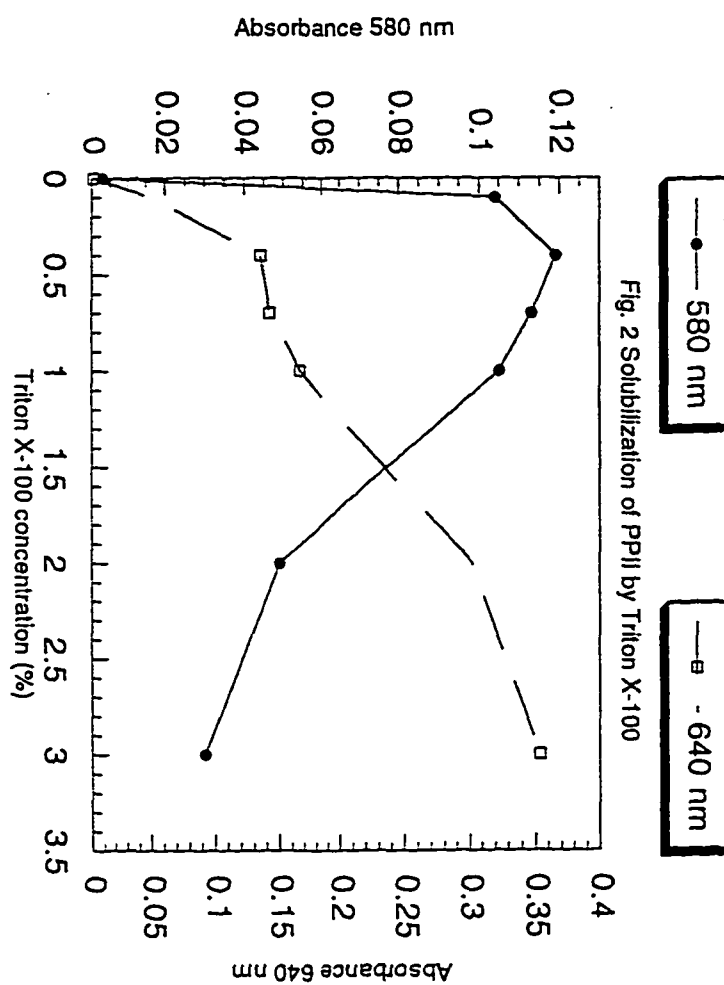


Figure 3

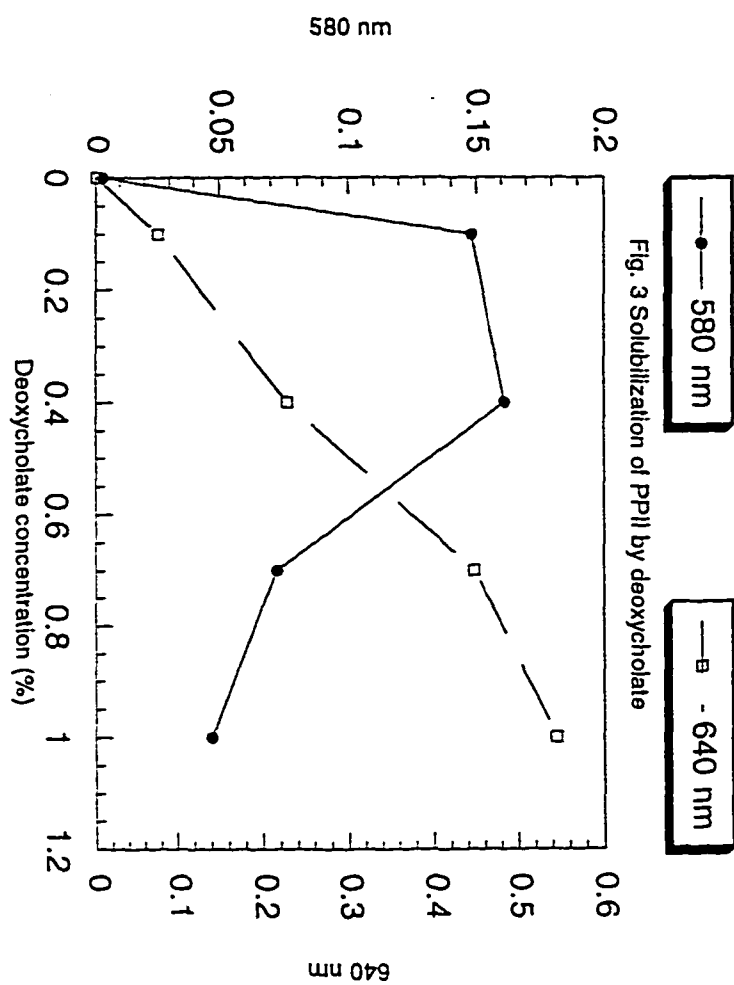


Figure 4

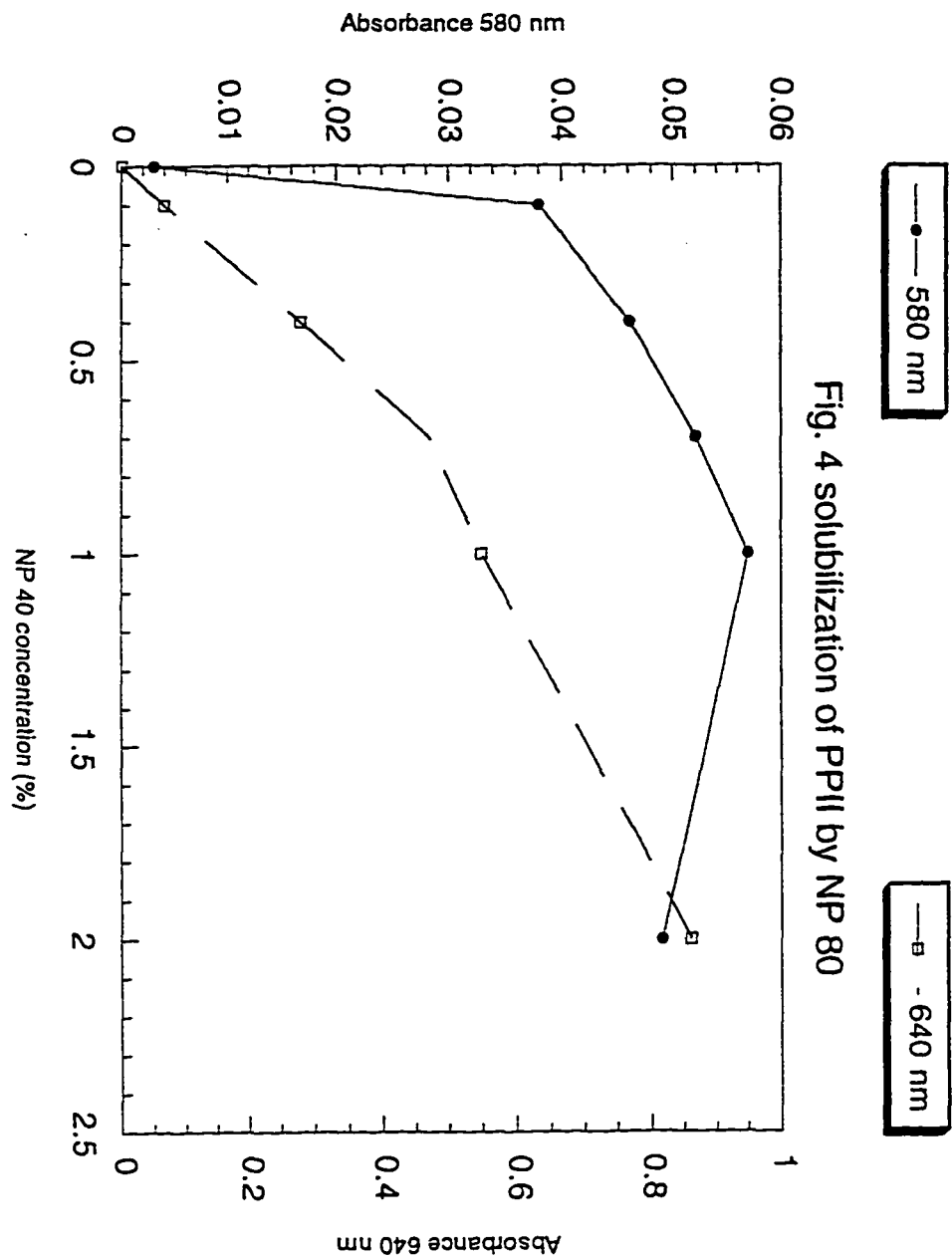


Figure 5

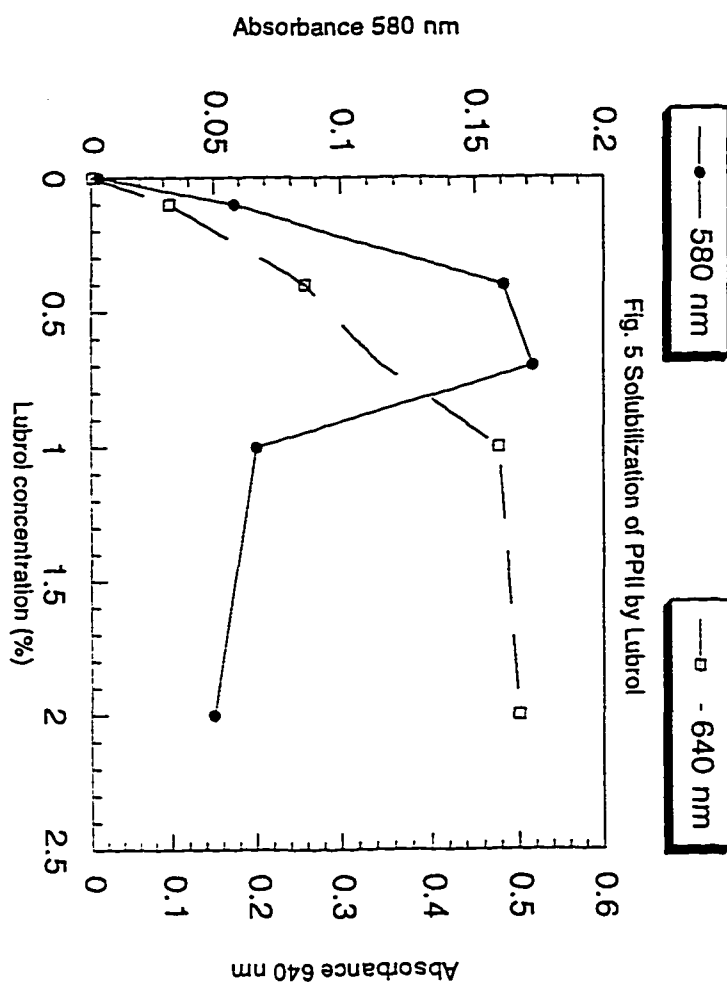


Figure 6

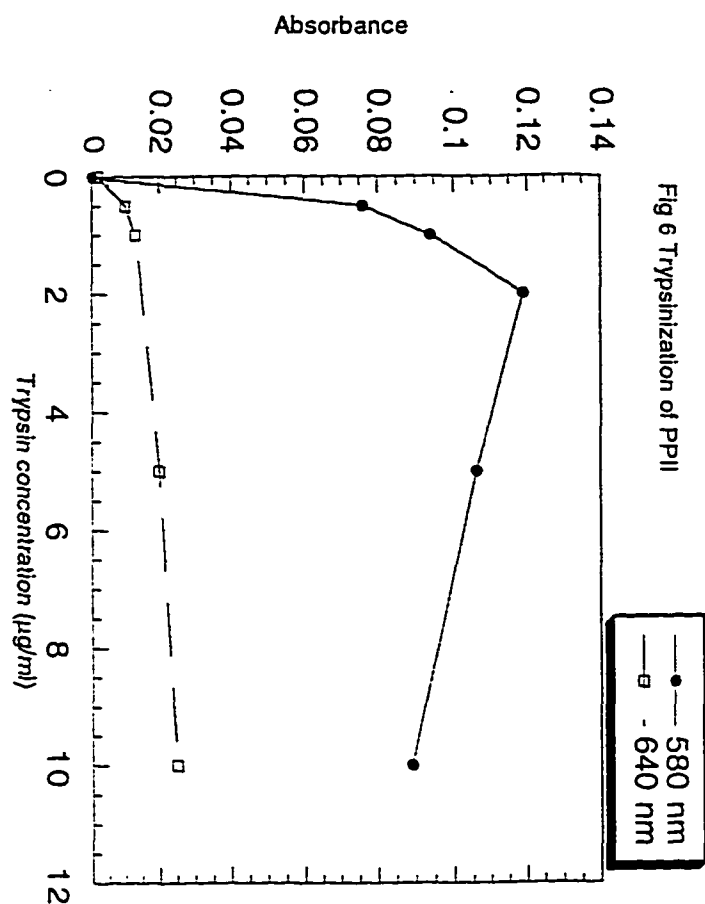


Figure 7

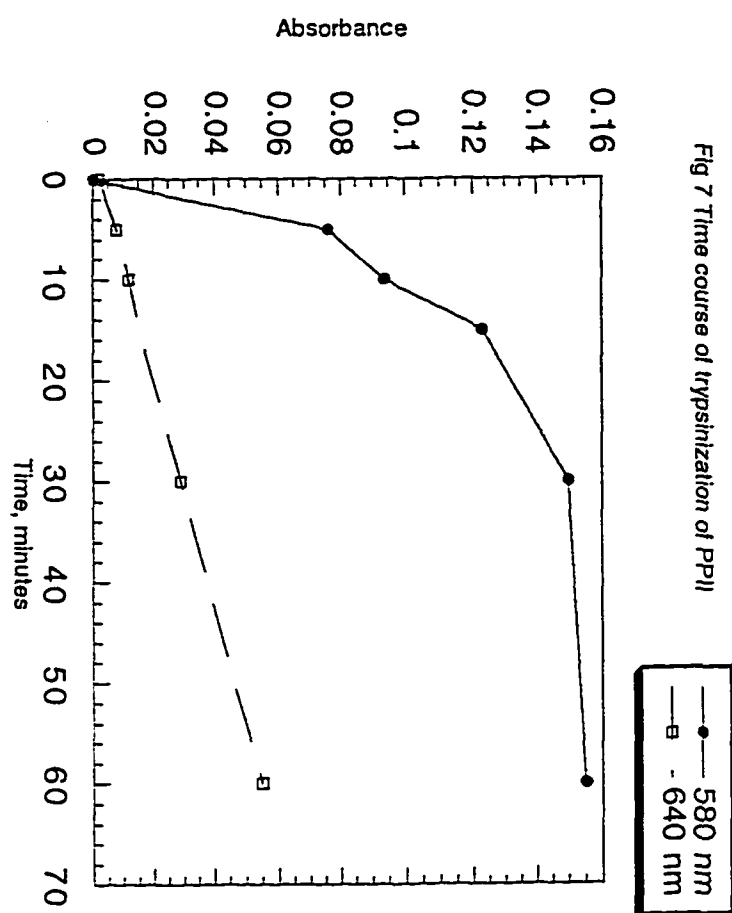


Figure 8

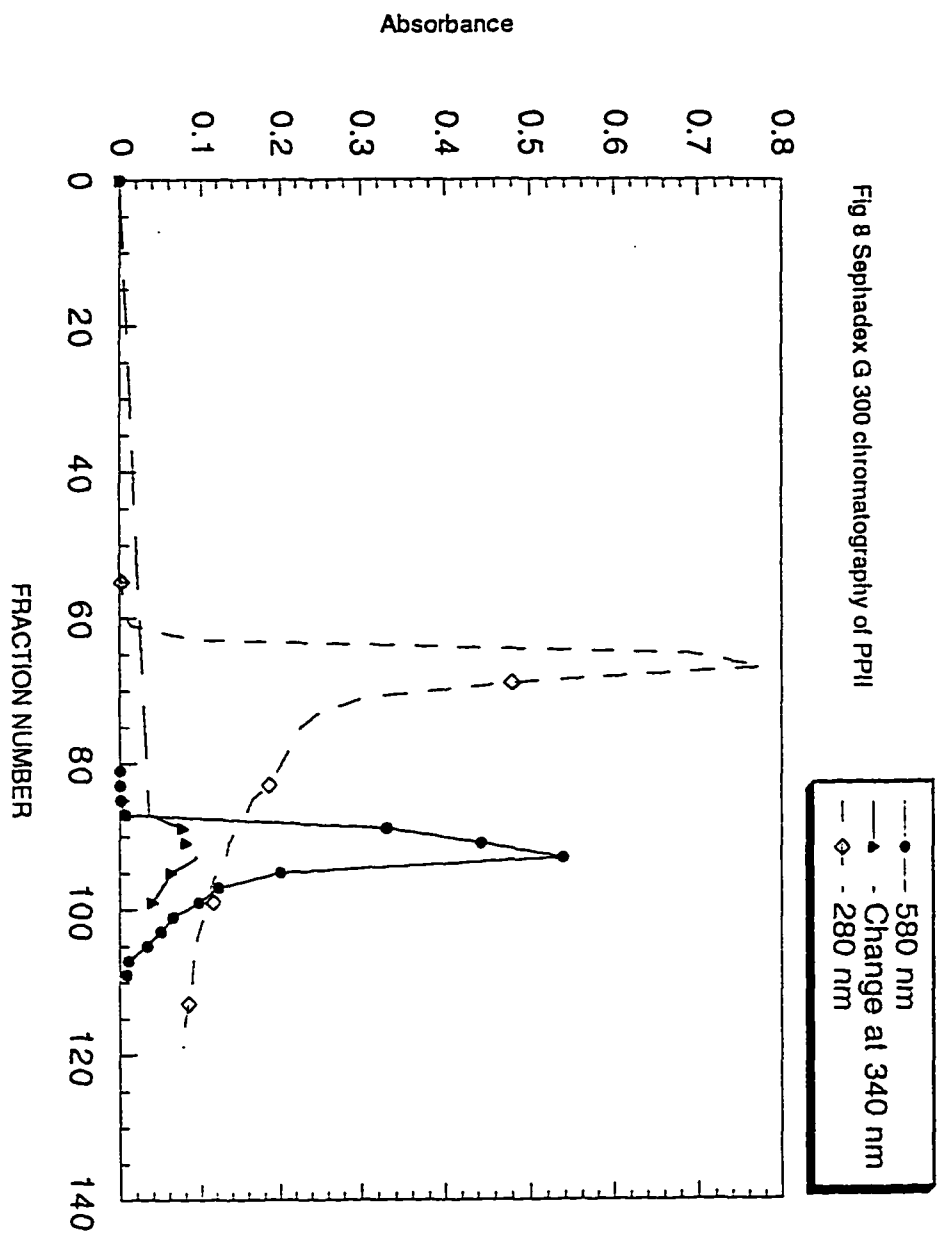


Figure 9

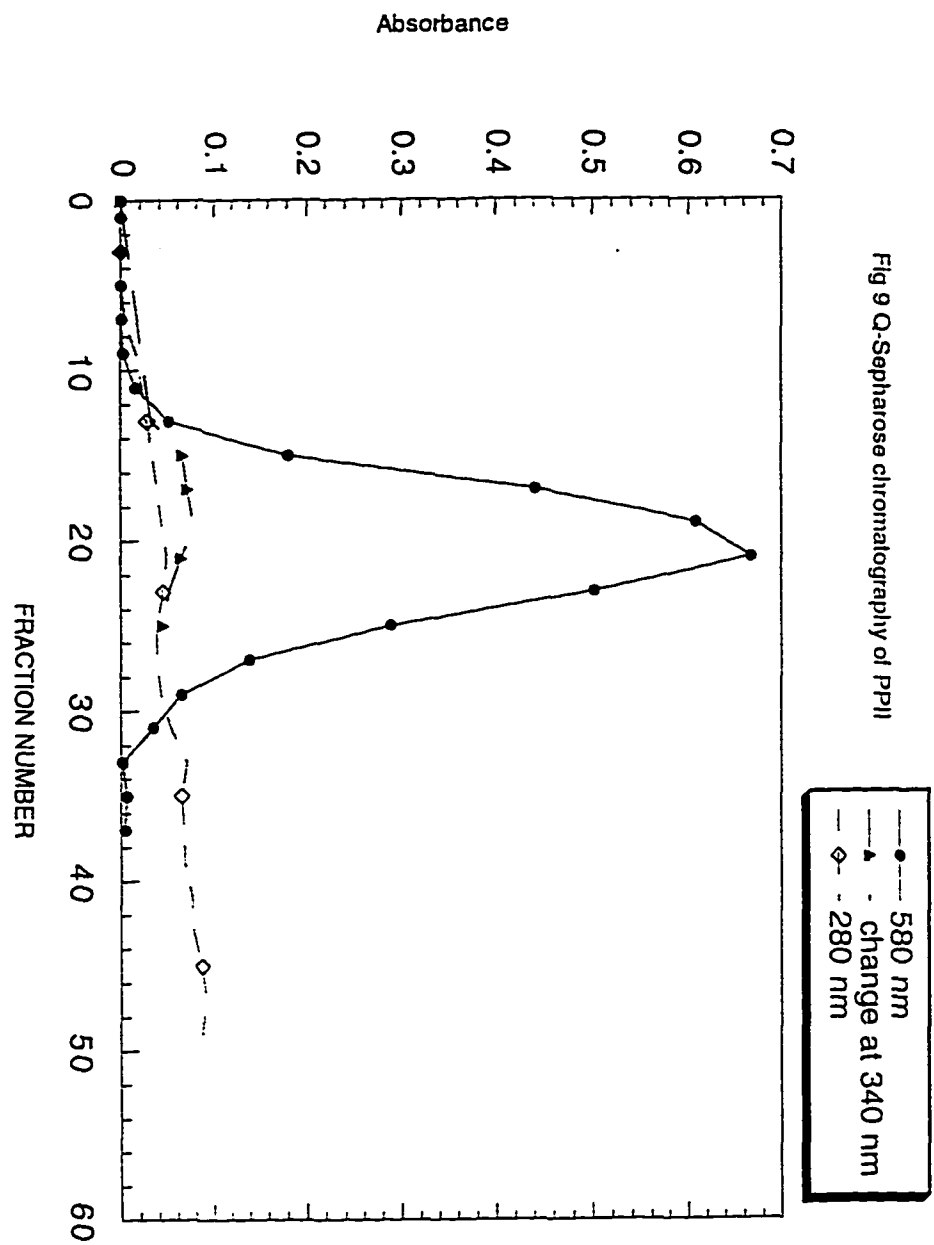


Figure 10

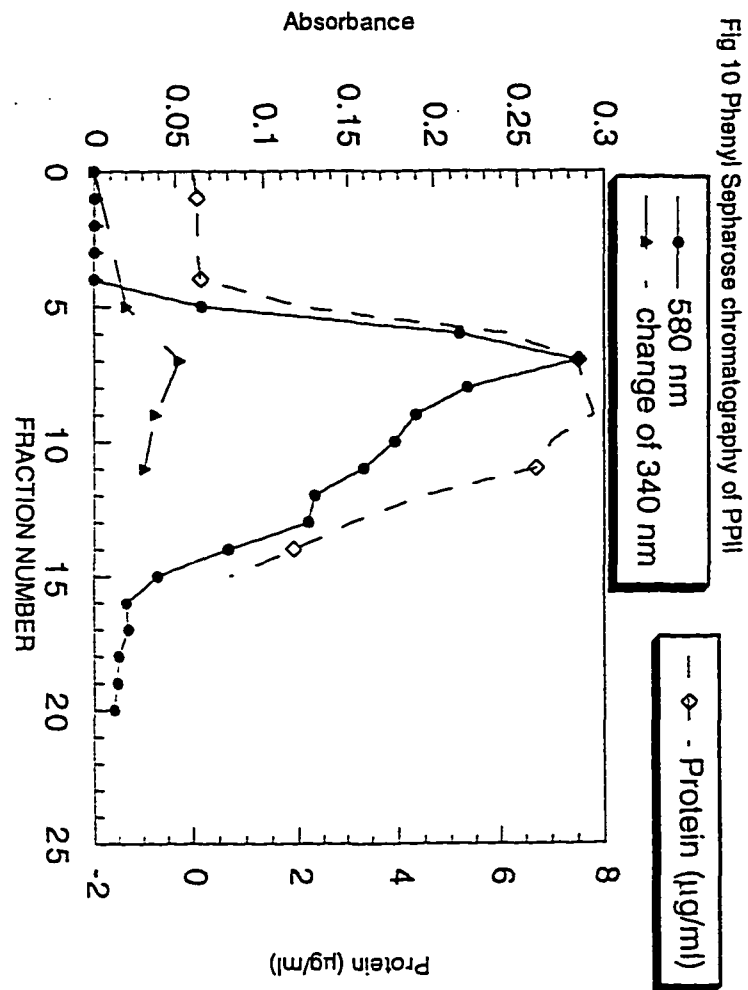


Figure 11

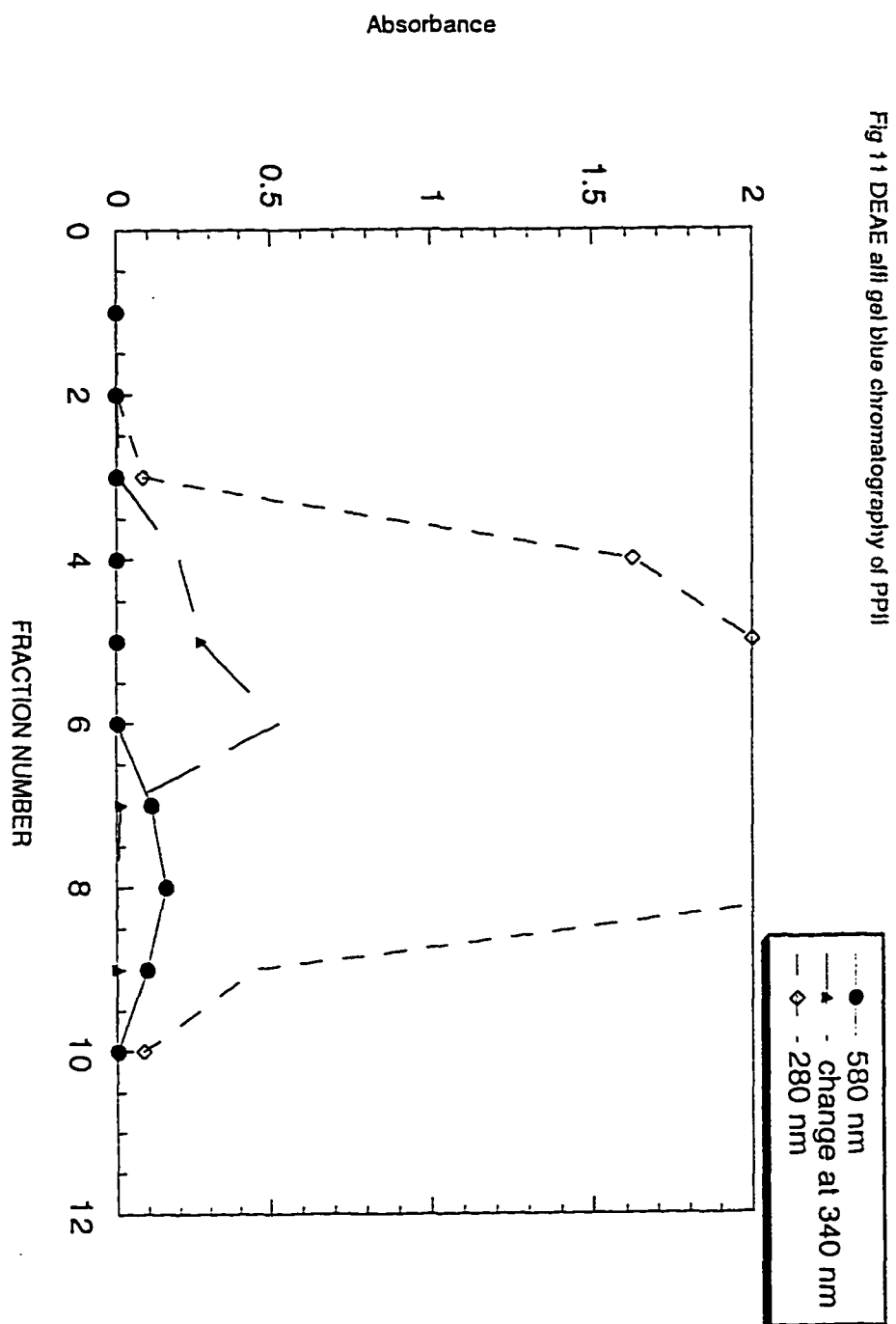


Figure 12

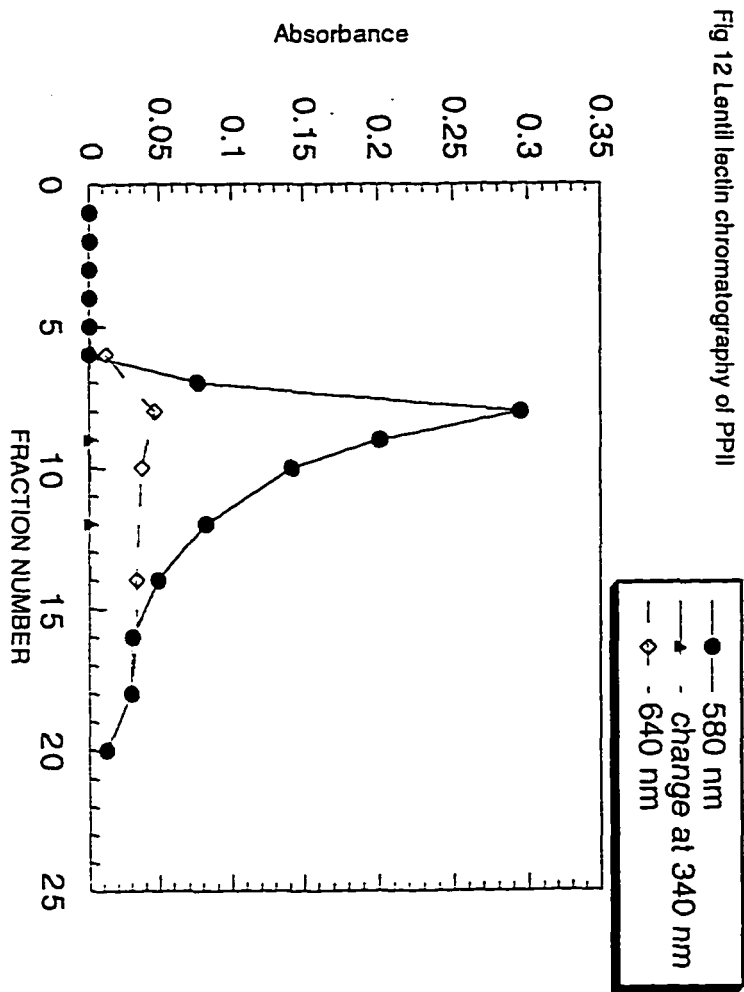


Figure 13

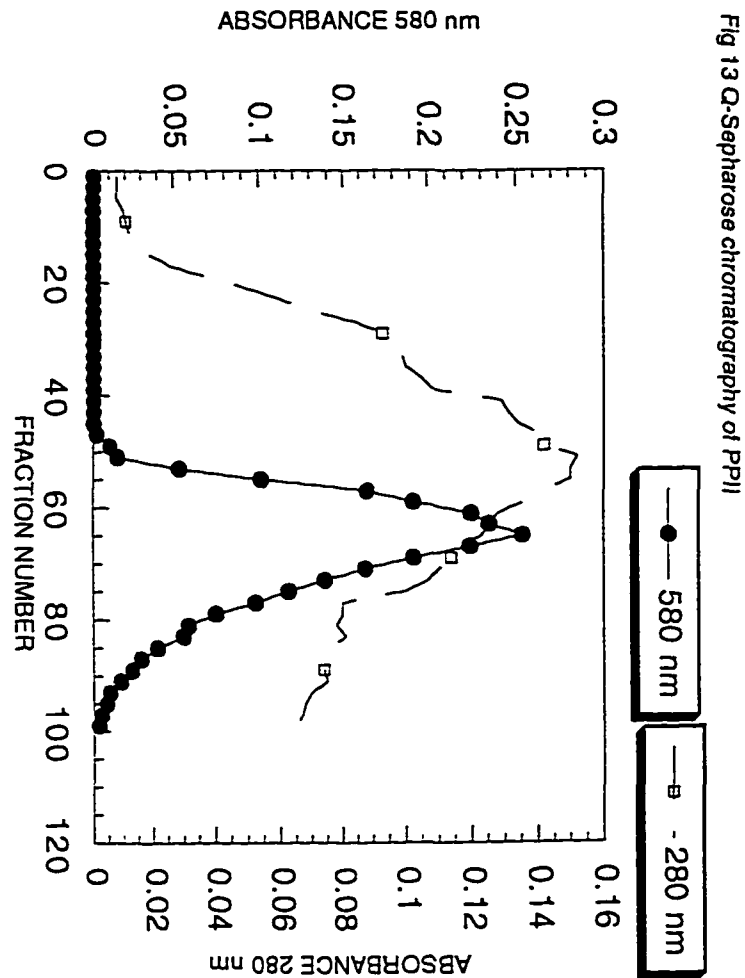


Figure 14

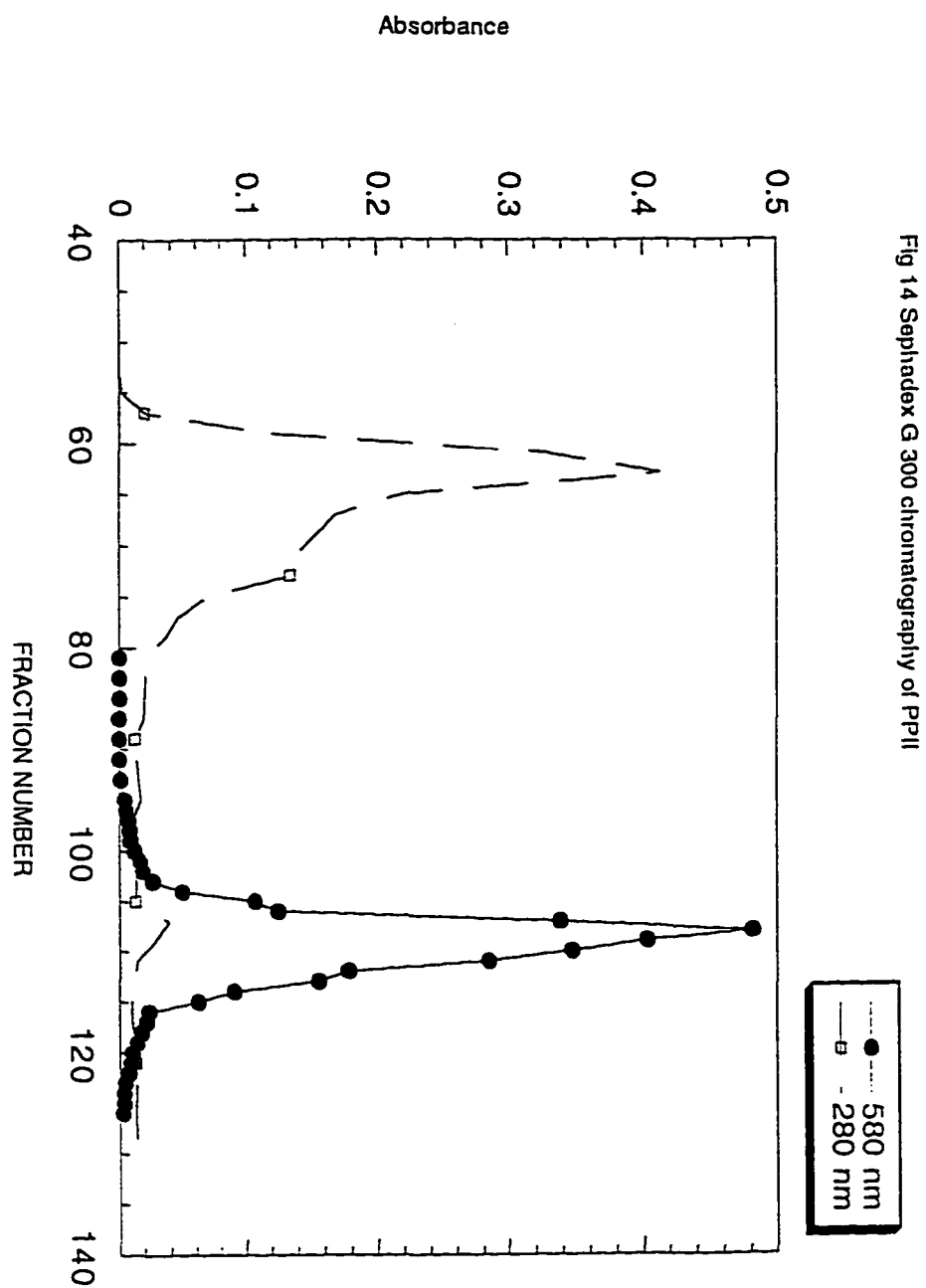


Figure 15

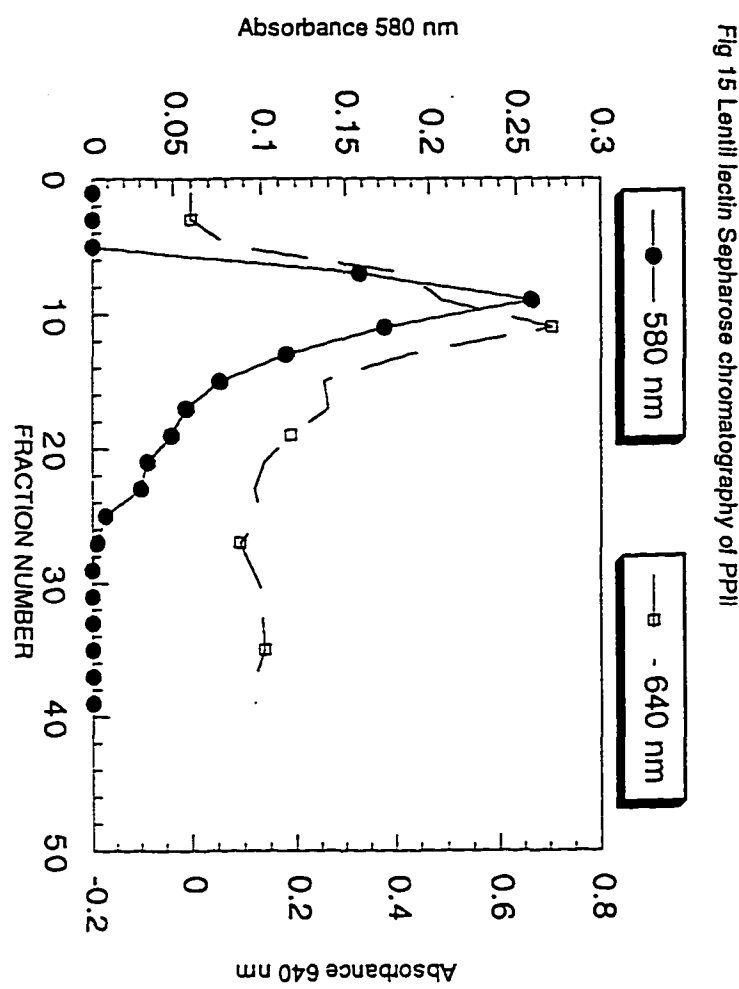


Figure 16

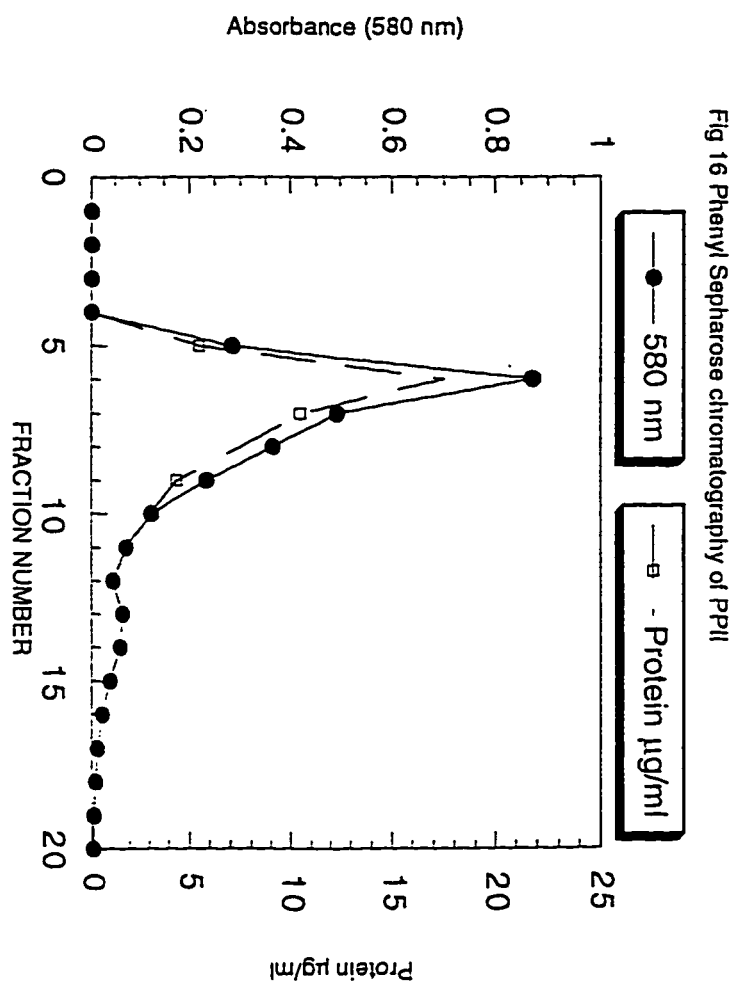


Figure 17

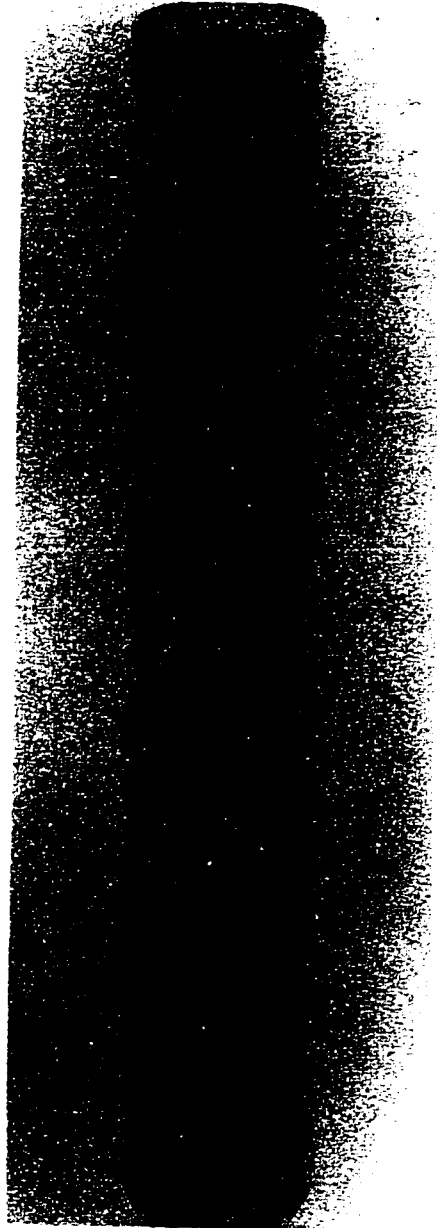


Figure 18

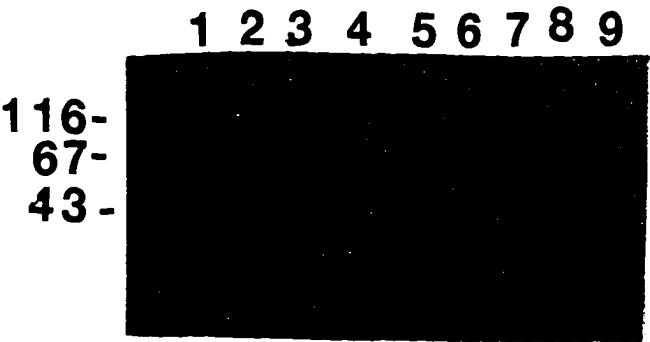


Figure 19

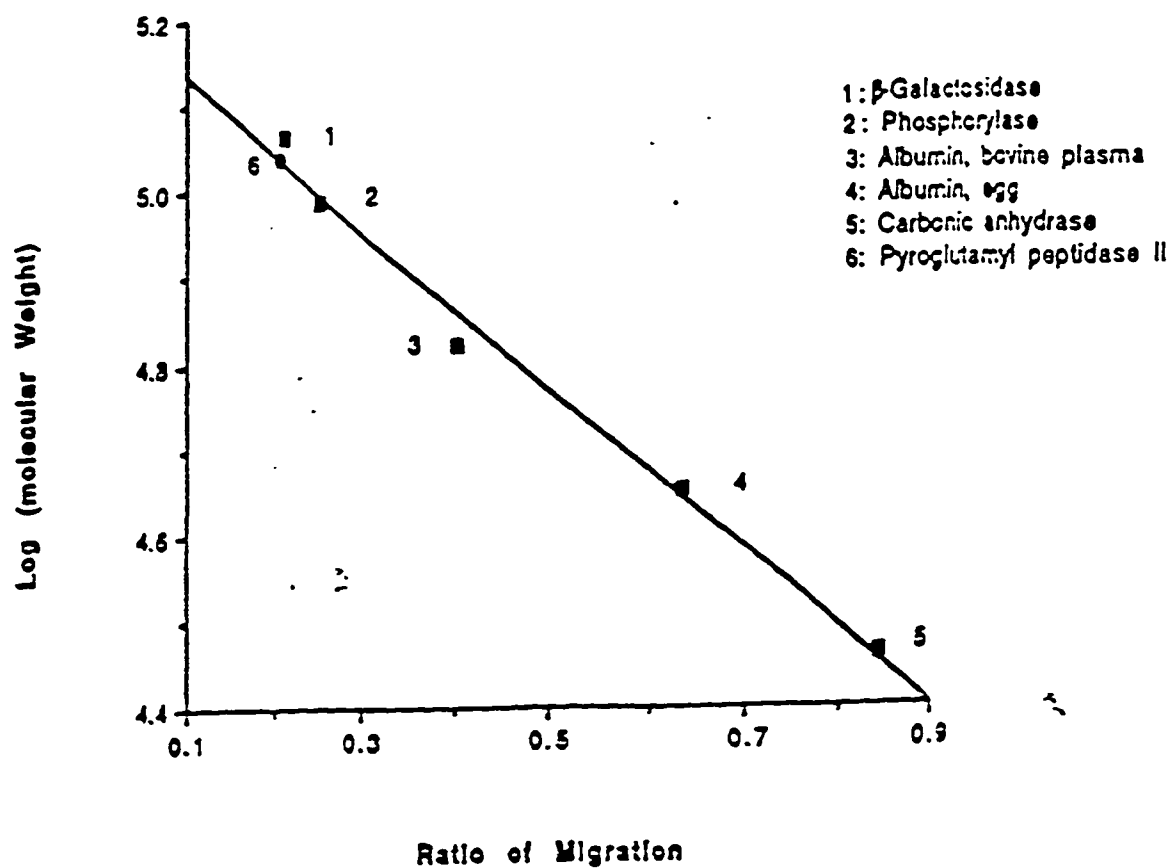
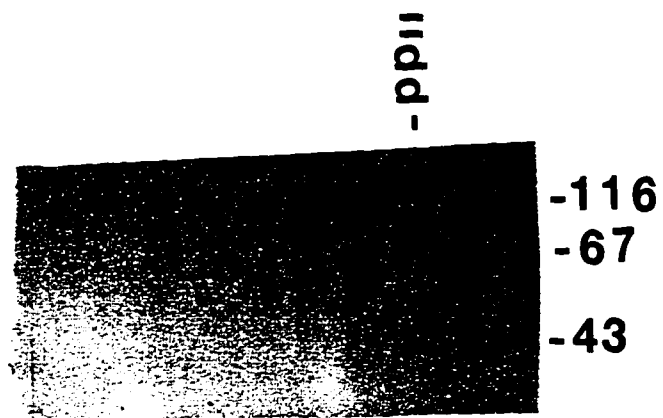


Figure 20



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Figure 21

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Figure 22

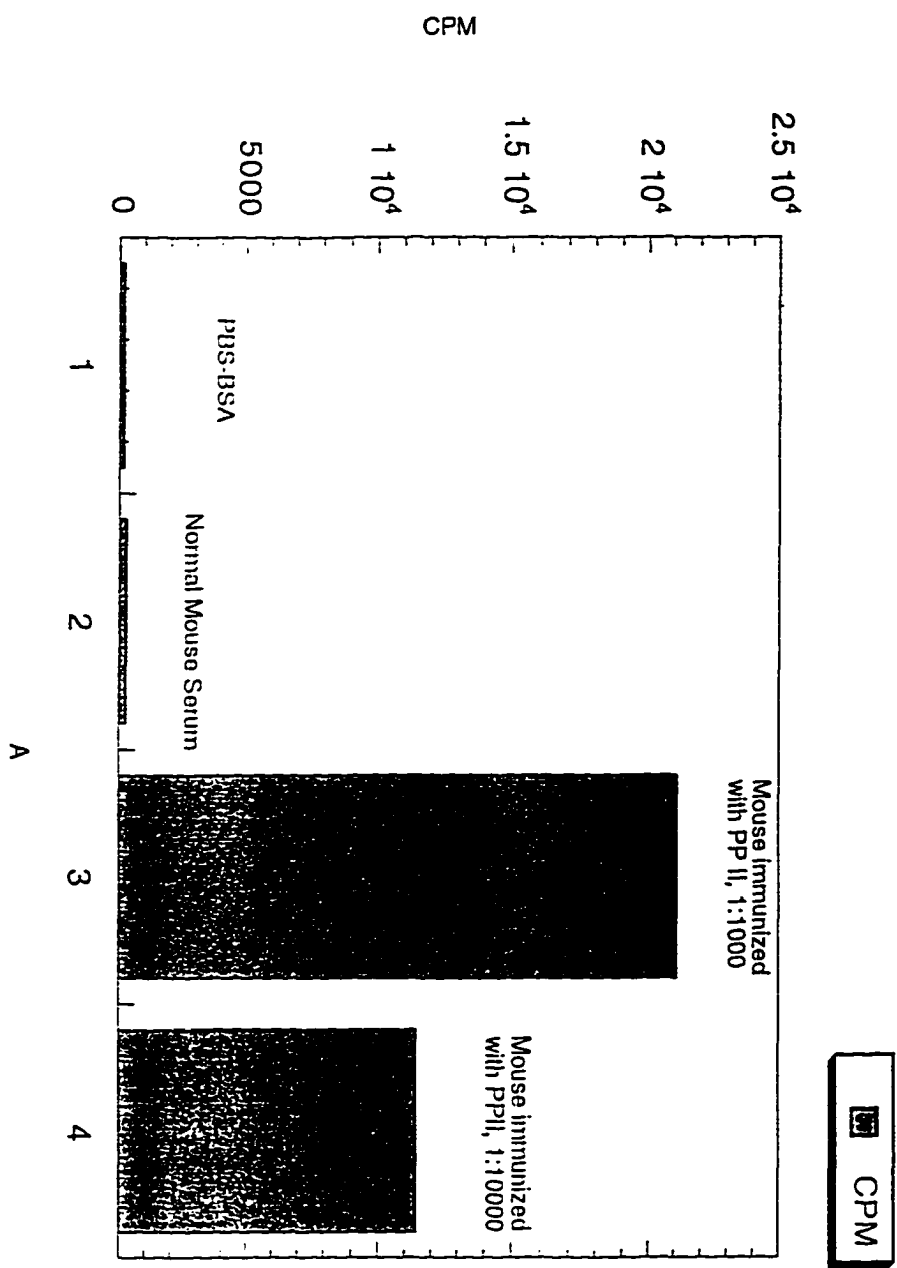


Table 1. Solubilization of Pyroglutamyl peptidase II from rabbit brain by several detergents.

Detergent	Total units of PP II Activity				
	Washed Pellet	Ultracentrif.	Sephadex G300	Q-Sepharose	Lentil lectin
CHAPS (0.7%)	15	31	11	5	0
Triton X-100 (0.4%)	11	16	3	0	
Deoxycholate (0.4%)	9	18	1		
NP-80 (1%)	10	7	0		
Lubrol-px (0.4%)	9	4	0		

Purification followed the procedure described in Methods, starting with 20-30 grams of rabbit brain for each detergent. The concentrations of detergents have been optimized as that solubilizing the highest specific activity of pyroglutamyl peptidase II. One unit of enzyme activity is defined as the release of 1 μ mol NA per hour from the substrate pGlu-His-Pro-NA.

Table 2. Purification of Pyroglutamyl peptidase II from rabbit brain using a strong trypsinization procedure (20 mg trypsin, 37 °C for 1 hr).

Steps	Volume (ml)	Protein (mg/ml)	Total Units	Specific Activity (unit/mg)	Yield (%)
Washed Pellet	250	10.5	45	0.017	100
Trypsinization	240	4.0	40	0.041	89
Acid Precipitation	55	2.5	22	0.13	49
Sephadex G300	75	0.27	18	0.923	40
Q-Sepharose	65	0.037	11	4.54	24
Affi gel blue	25	0.012	5.25	17.5	12
Phenyl Sepharose	12	0.003	2.7	75	6

Purification as described in Methods from 81 grams of rabbit brain. One unit of enzyme activity is defined as the release of 1 μ mol NA per hour from the substrate pGlu-His-Pro-NA.

Table 3. Purification of Pyroglutamyl peptidase II from rabbit brain following facile trypsinization (2 μ g/ml, 4 °C for 15 min).

Steps	Volume (ml)	Protein (mg/ml)	Total Units	Specific Activity (unit/mg)	Yield (%)
Washed Pellet	600	11.5	75	0.011	100
Trypsinization	500	3.6	60	0.033	80
Q-Sepharose	210	0.07	31	2.1	41
Sephadex G300	100	0.015	16	10.7	21
Lentil Lectin	50	0.008	8.5	21.2	11
Phenyl Sepharose	10	0.002	3.0	135	4

Purification started with 136 grams of rabbit brain. One unit of enzyme activity is defined as the release of 1 μ mol NA per hour from the substrate pGlu-His-Pro-NA.

Chapter 3

Distribution and Regulation of Pyroglutamyl Peptidase II Messenger
RNA Levels in Rats as Determined by a Quantitative Ribonuclease
Protection Assay

Abstract

We have established a reliable and quantitative solution hybridization ribonuclease protection assay for measurement of the mRNA of the specific thyrotropin-releasing hormone (TRH)-degrading enzyme pyroglutamyl peptidase II. Pyroglutamyl peptidase II mRNA levels were determined in major organs and brain regions of the rat. The highest level of pyroglutamyl peptidase II mRNA was found in brain (705 ± 47 fg/ μ g total RNA). Significant levels of the mRNA were also found in lung (136 ± 13 fg/ μ g) and pituitary (42 ± 2.3 fg/ μ g), whereas liver, heart, duodenum/pancreas, intestine, spleen, kidney, testis, and muscle contained lower but detectable amounts of pyroglutamyl peptidase II mRNA. In the brain the distribution of pyroglutamyl peptidase II mRNA was uneven with highest levels found in posterior cortex (1056 ± 83 fg/ μ g) and lowest in brain stem (129 ± 16 fg/ μ g). A single dose of T₃ markedly increased the level of pyroglutamyl peptidase II mRNA in the pituitary ($p < 0.01$), and in the liver ($p < 0.05$). A single dose of TRH had no significant effect on pyroglutamyl peptidase II mRNA levels. Elevation of pyroglutamyl peptidase II mRNA in liver by T₃ suggests that this organ is the source of the enzyme in serum.

A highly specific membrane-bound ectoenzyme termed pyroglutamyl peptidase II (EC 3.4.19.6) is believed to function to terminate the action of neuronally released thyrotropin-releasing hormone (TRH) (Wilk, 1986; Bauer, 1995; O'Leary and O'Connor, 1995). This enzyme is located on adenohypophyseal cell membranes (Vargas et al., 1987) and brain synaptosomal membranes (O'Connor and O'Cuinn, 1984; Torres et al., 1986). Pyroglutamyl peptidase II cleaves the pyroglutamyl-histidyl bond of TRH (pGlu-His-ProNH₂) but not the pyroglutamyl-histidyl bond of GnRH (O'Connor and O'Cuinn, 1985). Studies with specific inhibitors have shown that pyroglutamyl peptidase II is clearly distinct from the less specific cytosolic enzyme pyroglutamyl peptidase I (EC 3.4.19.3) (Friedman and Wilk, 1986). An activity present in rat serum and termed "thyroliberinase" because of its high substrate specificity (Taylor and Dixon, 1978; Bauer and Nowak, 1979) is most likely pyroglutamyl peptidase II (Friedman and Wilk, 1985). Pyroglutamyl peptidase II, a homodimer of molecular weight of 230,000 (Bauer, 1994), has been purified from guinea pig brain (O'Connor and O'Cuinn, 1985), rabbit brain (Wilk and Wilk, 1989), and rat and porcine brain (Bauer, 1994). The cDNA encoding the rat brain enzyme has been cloned (Schauder et al., 1994).

Determination of the distribution of pyroglutamyl peptidase II is of importance for understanding its physiological significance. Measurement of enzymatic activity revealed that the highest concentration of pyroglutamyl peptidase II is found in the brain where its regional distribution is uneven (Friedman and Wilk, 1986; Vargas et al., 1992), with significant activity also present in lung and pituitary (Friedman and Wilk, 1986). Northern analysis demonstrated that the tissue distribution of mRNA levels paralleled the previously described distribution of enzymatic activity (Schauder et al., 1994).

The regulation of pyroglutamyl peptidase II activity has attracted considerable interest. Thyroid hormone was demonstrated to increase pyroglutamyl peptidase II (thyroliberinase) activity in rat serum (White et al., 1976), adenohypophysis (Bauer, 1987), frontal cortex (Suen and Wilk, 1989), and liver (Scharfmann et al., 1990). The time course of the effect of thyroid hormone is consistent with a role for this hormone in increasing synthesis of pyroglutamyl peptidase II. More recently it has been shown that the pituitary levels of pyroglutamyl peptidase mRNA are markedly increased by thyroid hormone treatment with a maximum effect seen 6-20 h after administration (Schomburg and Bauer, 1995). In primary rat pituitary cultures, pyroglutamyl peptidase II activity is also increased by thyroid hormone (Bauer et al., 1990). Pyroglutamyl peptidase II activity is markedly down-regulated in the Y-79 retinoblastoma cell by phorbol ester (Suen and Wilk, 1990), and down-regulated by TRH in adenohypophyseal cultures (Vargas et al., 1994).

We were interested in further exploring the distribution and regulation of pyroglutamyl peptidase II mRNA levels in brain regions, pituitary and other organs. To overcome the difficulty of the low abundance of pyroglutamyl peptidase II mRNA, we have established a sensitive, reliable and quantitative solution hybridization ribonuclease protection assay based on the sequence of the recently cloned cDNA (Schauder et al., 1994). Our studies document the uneven distribution of pyroglutamyl peptidase II mRNA levels in rat major organs and brain regions, and explore the acute effects of treatment with thyroid hormone and TRH.

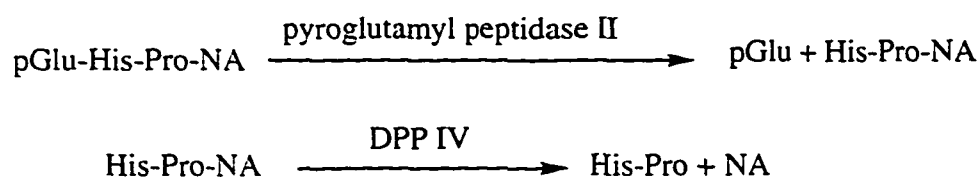
Experimental Procedures

Animal Experiments

Male Sprague-Dawley rats weighing 225-250 grams, were housed on a 12-12 h light-dark schedule and allowed free access to food and water. The distribution studies used 6 untreated rats. Four additional groups of 4-8 rats each were used for studies on regulation. One group received a single subcutaneous injection of 100 µg T₃ in 10 mM NaOH. A second group was administered a single intravenous injection of 300 µg TRH in 0.9% NaCl. Two control groups were administered vehicle only. All rats were sacrificed by carbon dioxide asphyxiation 16 h after injection. Immediately after sacrifice, the brain and other organs of interest were rapidly removed, and the brain regions dissected. Tissue samples were frozen on dry ice, and stored at -80 °C before processing for total RNA extraction or enzymatic assays. The animal experiments were approved by our Institutional Animal Care and Use Committee.

Measurement of enzymatic activity of pyroglutamyl peptidase II

Pyroglutamyl peptidase II activity was measured by a modification of the previously described method (Friedman and Wilk, 1986). Briefly, enzymatic activity was determined with the synthetic substrate pGlu-His-Pro-naphthylamide (NA) in a coupled assay in the presence of an excess amount of dipeptidyl peptidase IV (DPP IV), purified as described (Li et al., 1995).



The reaction mixture consisted of 50 mM sodium phosphate buffer (pH 7.5), 5 μ l of DPP IV (1 μ g protein) and 5 μ l of substrate (10 mM), 5 μ l PCMB (0.4 mM) and tissue sample, in a total volume of 125 μ l. Tissue samples were immediately frozen on dry ice and stored at -80 °C. At assay, the samples were thawed, homogenized in 50 mM phosphate buffer (pH 7.5) and washed 5 times. PCMB was used to inhibit the activities of prolyl oligopeptidase (Orlowski et al., 1979) and pyroglutamyl peptidase I (Szewczuk and Kwiatowska, 1970). Tubes were incubated at 37° C for 30 min to 2h, and the reaction terminated by addition of 125 μ l of 10% TCA. Free NA was diazotized and quantitated by a colorimetric reaction. The absorbance of the chromogen was read at 580 nm. One unit of enzyme activity was defined as the amount of enzyme releasing 1 nmol NA/h.

Isolation of cytoplasmic RNA

Total cytoplasmic RNA was isolated from the dissected brain regions, pituitary and peripheral organs by a minor modification of the method described (Lazar and Blum, 1992). Briefly, individual tissue was removed from -80 °C storage, homogenized with 500 μ l ice-cold lysis buffer (10 mM Tris-HCl, pH 7.4, 1.5 mM MgCl₂, 0.5% Nonidet P-40, 0.25% sodium deoxycholate, 0.3 M sucrose). Lysates were layered over sucrose cushions (10 mM Tris-HCl, pH 7.4, 1.5 mM MgCl₂, 0.4 M sucrose) and centrifuged at 5000 g for 10 min at 4 °C. The upper phases (600 μ l) were transferred to new tubes and 60 μ l 10X SET (10% SDS, 50 mM EDTA, 10 mM Tris-HCl pH 8) and 6 μ l proteinase K (10 mg/ml) were added. After incubation at 45 °C for 1 hr, 66 μ l 3M ammonium acetate, 300 μ l phenol and 300 μ l chloroform were added. The tubes were vortexed and centrifuged. After transfer of the aqueous phases to new tubes,

730 μ l of cold isopropanol was added and the tubes stored at -20 $^{\circ}$ C overnight. The RNA recovered by centrifuging at 16 000 g for 10 min, was washed with 70% ethanol, dried, and resuspended in diethylpyrocarbonate-treated deionized water. RNA concentration was determined by A₂₆₀. The integrity of RNA was examined after agarose gel electrophoresis in gels containing ethidium bromide. The chemicals and solutions employed in the experiments with RNA were tested as RNase free.

Generation of DNA templates for in vitro transcription of RNA standards and ³²P-labeled antisense riboprobes.

An cDNA sequence of 483 nucleotides of pyroglutamyl peptidase II, from nucleotides 287 to 784 was chosen to be amplified by PCR. The sequence was selected on the basis of low homology to other known sequences in the data bank. A sense primer and an antisense primer were synthesized in the Molecular Biology Core Facility. PCR reactions were performed for 30 cycles with a Stratagene rat brain cDNA library as template in the presence of Taq DNA polymerase. The reaction temperatures were set as 95 $^{\circ}$ C for denaturation, 60 $^{\circ}$ C for annealing, and 72 $^{\circ}$ C for extension. A negative control consisted of the reaction mixture without the template. A major PCR product was a band slightly smaller than 500 bps. It was ligated into a TA cloning plasmid vector PCRTM II flanked by T7 and SP6 RNA polymerase promoter sites (Fig. 1a). The plasmid DNA was transformed in competent *E. Coli*, and grown on kanamycin and ampicillin agar plates containing X-gal. White colonies containing inserts were picked up for plasmid DNA miniprep. Plasmid DNA was digested with EcoRI to examine the size of inserts. The potential clones were examined by their restriction patterns with EcoRI and SmaI, which resulted in 2 fractions of correct sizes. The sequence of the correct clone was

confirmed by plasmid DNA sequencing. The orientation was determined by the digestion patterns with the restriction enzymes Hind III and Sma I (Fig 1a).

In preparation for *in vitro* transcription reactions, the plasmid was enzymatically restricted 5' within the insert with Hinc II for the antisense riboprobe. This generates a riboprobe of 277 bps, containing 209 bps of the pyroglutamyl peptidase II cDNA fragment and 68 bps of plasmid DNA (Fig. 1b). For the sense-strand RNA standard, the plasmid was enzymatically restricted 3' within the multiple cloning site with Xho I (Fig. 1c). The linearized plasmid DNAs were purified by agarose gel electrophoresis and recovered by the GeneClean DNA isolation technique (Bio 101).

Synthesis of ^{32}P -labelled antisense riboprobe and unlabelled RNA standards.

The synthesis of a ^{32}P -labelled antisense riboprobe of 277 bps was performed with the aid of a Gemini kit. The reaction mixture contained 15 μl ^{32}P UTP (800 Ci/mmol, New England Nuclear, dried in a vacuum dryer), 2.5 μl dH₂O, 2 μl 5X transcription buffer, 1 μl 100 mM DTT, 0.5 μl RNasin, 1 μl 10 mM ATP, 1 μl 10 mM CTP, 1 μl 10 mM GTP, and 0.5 μl linearized DNA template (0.5 mg/ml). The *in vitro* transcription occurred after the addition of 0.5 μl RNA polymerase T7 (5U/ml). The mixture was incubated for 30 min at 37 °C. The plasmid DNA template was then digested by adding 90 μl H₂O, 5 μl 1M Tris-HCl (pH 8), 1 μl 1M MgCl₂, 1 μl RNasin and 1 μl DNase (ultrapure, 20 U/ml) and incubating for 30 min at 37 °C. The synthesized riboprobe was purified by chromatography on a Sephadex G 100 column. The quality of the probe was examined by acrylamide gel electrophoresis. The amount of probe synthesized was calculated by measuring the incorporated CPM of the aliquot.

An RNA standard was synthesized and purified in a similar way. In this case, the reaction consisted of 16.5 μl dH₂O, 10 μl 5X transcription buffer, 5 μl

100 mM DTT, 2 μ l RNasin, 2.5 μ l 10 mM ATP, 2.5 μ l 10 mM CTP, 2.5 μ l 10 mM GTP, 2.5 μ l 10 mM UTP, 0.5 μ l ^{32} P UTP, and 2.5 μ l linearized DNA template (0.5 mg/ml). The *in vitro* transcription occurred after the addition of 1 μ l RNA polymerase SP6 (25 U/ml). The amount of RNA standard was calculated, aliquoted and stored at -80 °C.

Solution hybridization ribonuclease protection assay.

RNA standards consisted of increasing quantities of *in vitro* synthesized sense-stand RNA, generally from 0 pg to 16 pg. Total cytosolic RNA of individual tissue samples ranged from 10 μ g to 80 μ g. RNA standards and RNA samples were dried down in a vacuum speed dryer, resuspended in 30 μ l hybridization buffer (80% formamide, 0.4 M NaCl, 1 mM EDTA, 40 mM PIPES and 2.5 mg/ml yeast total RNA) containing 0.5 -1 μ l freshly synthesized riboprobe (100-200 pg)). Samples were heated at 85 °C for 5 min, and then incubated at 45 °C overnight (16 h). Samples were treated with 300 μ l of 1X RNase buffer (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, pH 8, 0.3M NaCl) containing 40 μ g/ml RNase A and 2 μ g/ml of RNase T1, and incubated at 30 °C for 1 h. Proteinase K (10 mg/ml) and 10% SDS were added (5 μ l of each) and the samples incubated at 45 °C for 15 min. The samples were extracted with phenol-chloroform, and the aqueous phase treated with isopropanol in the presence of 10 μ g total yeast RNA. RNA:RNA hybrid pellets were recovered by centrifugation, washed with 70% ethanol, resuspended in 8 μ l gel loading buffer, and analyzed in 4% non-denaturing acrylamide gels. The gels were dried, and placed onto a phosphorimager screen (Molecular Dynamics) for 16-24 h. The volumes of hybridized bands were quantitated by a computer assisted phosphorimager system. The computer software Statview was used to calculate the standard curve and the sample amounts. Because of the size difference

between the hybrids with synthesized standard (+) RNA and tissue isolated mRNA, a correction factor, $277/209$, was applied to the calculation of sample pyroglutamyl peptidase II mRNA.

Results

Detection of pyroglutamyl peptidase II mRNA

A solution hybridization ribonuclease protection assay was developed to detect and quantitate pyroglutamyl peptidase II mRNA in rat organs. The hybrid of the riboprobe with increasing quantities of the sense-strand RNA standard is shown in Fig. 2A. Total yeast RNA, serving as a negative control, was tested for its inability to protect the pyroglutamyl peptidase II riboprobe from ribonuclease digestion. As shown in lane 8 of Fig. 2A, the hybrid was absent in the presence of total yeast RNA alone, confirming the complete removal of all unhybridized radiolabeled riboprobe. There was also an excellent linear correlation between the volumes of the hybrids and increasing quantities of sense strand RNA (Fig. 2B).

Distribution of pyroglutamyl peptidase II mRNA in rat organs

Pyroglutamyl peptidase II mRNA was detected in rat brain, pituitary, lung, liver, heart, duodenum/pancreas, intestine, spleen, kidney, testis, heart and muscle. The highest level of mRNA of pyroglutamyl peptidase II was found in brain (705 ± 47 fg/ μ g total RNA). Significant levels of the mRNA were found in lung (116 ± 13 fg/ μ g) and pituitary (42 ± 2 fg/ μ g). Other organs contained lower but measurable amounts of pyroglutamyl peptidase II mRNA (Fig 3; Table 1). Determination of pyroglutamyl peptidase II enzymatic activity yielded values in agreement with the previously published results (Friedman and Wilk, 1986) (Table 1). The mRNA levels of pyroglutamyl peptidase II correlated with the distribution of the enzymatic activities in rat major organs ($r = .989$).

Distribution of pyroglutamyl peptidase II mRNA in rat brain regions

The volumes of hybridized bands of pyroglutamyl peptidase II mRNA were highly correlated with amount of brain total RNA (Fig. 4A). The distribution of pyroglutamyl peptidase II mRNA in the brain was uneven (Fig. 4B). The most abundant amount was found in posterior cortex (1056 ± 83 fg/ μ g), followed by hippocampus, cerebellum, frontal cortex, olfactory bulb, amygdala, striatum, thalamus, hypothalamus, and lowest in brain stem (129 ± 16 fg/ μ g) (Table 2). Enzymatic activities in the brain regions were in agreement with our previously published studies (Friedman and Wilk, 1986) (Table 2), and correlated with mRNA levels with the exception of the cerebellum. In the cerebellum, enzymatic activity is relatively low, whereas the mRNA level is relatively high. Omitting the cerebellum, enzymatic activity and mRNA levels were highly correlated ($r=0.892$).

Effect of T3 and TRH on pyroglutamyl peptidase II mRNA in rat organs and brain regions

A single dose of T3 markedly increased the level of pyroglutamyl peptidase II mRNA and enzyme activity in the pituitary ($p<0.01$) but not in the frontal cortex (Fig. 5; Tables 3,4,5,6). A significant increase in pyroglutamyl peptidase II mRNA and enzyme activity was also observed in the liver following treatment with T3 ($p<0.05$) but not in the lung (Fig. 6; Table 3,5). T3 did not significantly change pyroglutamyl peptidase II mRNA levels in other peripheral organs or in whole brain (Table 3). Intravenously administered TRH had no significant effect on pyroglutamyl peptidase II mRNA level or enzyme activity in any tissue examined although a small tendency for a decrease in pituitary mRNA was noticed (Table 3,5). In all brain regions examined, neither T3 nor TRH had any effect on pyroglutamyl peptidase II mRNA levels or enzyme activity (Table 4,6).

Discussion

It is generally agreed that TRH not only functions as a hypothalamic hypophysiotrophic hormone (Schally et al., 1969), but also as a neurotransmitter/modulator in extrahypothalamic areas (Prasad, 1987). Pharmacological administration of TRH and its analogs have produced a number of centrally mediated effects (Horita et al., 1986). TRH was observed to have neurotropic (Banda et al., 1989) and antiataxic effects (Matsui et al., 1994), and its use proposed as treatment for brain and spinal trauma (Faden and Salzman, 1992). If TRH functions as a neurotransmitter, an effective mechanism must exist for termination of its action. *In vitro* studies have characterized three TRH degrading enzymes. Two of these, prolyl oligopeptidase and pyroglutamyl peptidase I, however, are cytosolic, widely distributed, and degrade many neuropeptides (Wilk, 1986; Bauer and Kleinkauf, 1980; Browne and O'Cuinn, 1983). Furthermore, inhibition of these two enzymes separately or in combination does not increase the recovery of TRH released from brain slices by K⁺ depolarization (Mendez et al., 1990). Therefore, they are unlikely to be the enzymes controlling the inactivation of neuronally released TRH. The third enzyme, pyroglutamyl peptidase II, on the basis of its high substrate specificity, active site orientation, synaptosomal location and on the basis of functional studies (Charli et al., 1989), most likely provides a specific and efficient mechanism for inactivation of neuronally released TRH (Wilk, 1986; Bauer, 1995). This agrees with the principle that the biological actions of neuropeptides are terminated or transformed by proteolytic enzymes, and thus the bioavailability of neuronally released peptides is predominantly controlled by peptide degrading ectoenzymes located on synaptosomal membranes (Molineaux and Wilk, 1991).

The distribution of pyroglutamyl peptidase II mRNA in major rat organs was previously determined by Northern hybridization (Schauder et al., 1994). We have used a highly sensitive solution hybridization ribonuclease protection assay to quantitate the gene expression of pyroglutamyl peptidase II in brain regions and peripheral organs. These studies provide the first quantitative data on pyroglutamyl peptidase II mRNA levels in rat brain regions, and the most complete description of pyroglutamyl peptidase II mRNA levels in peripheral organs. The information obtained supplements earlier studies measuring enzyme activity, and is of value for future *in situ* hybridization localization. The levels of pyroglutamyl peptidase II mRNA in rat major organs are highly correlated with levels of enzymatic activity. The highest level of pyroglutamyl peptidase II gene expression is in the central nervous system. This is consistent with a role for TRH as a putative neurotransmitter or modulator in the central nervous system since an adequate inactivating mechanism for neuronally released TRH is provided. The distribution of pyroglutamyl peptidase II mRNA in brain does not correlate with the regional levels of TRH where TRH levels are highest in hypothalamus and lowest in cerebellum (Winokur and Utiger, 1974). Neither is there a correlation with the distribution of TRH binding sites where density of receptor binding is highest in amygdala and lowest in cortex (Sharif, 1989) ($r = 0.29$, data not shown). More information concerning the comparative distribution of elements of the putative TRH neuronal system await the results of *in situ* hybridization localization studies of pyroglutamyl peptidase II mRNA.

Within the brain, regional mRNA distribution correlates with enzymatic activity with the exception of the cerebellum. In the cerebellum, the enzymatic activity of pyroglutamyl peptidase II is relatively low whereas its mRNA level is relatively high. We have not been able to demonstrate the presence of an endogenous inhibitor in the cerebellum (Lin, unpublished observations).

Interestingly a similar situation is found for TRH receptors in the cerebellum, where the receptor number is low, whereas its mRNA is relatively high (Kaji et al., 1993). It is of interest that administration of TRH produces anti-ataxia effects, believed to be due to an action on the cerebellum (Matsui et al., 1994). The significance of the presence of relatively high levels of mRNA's of pyroglutamyl peptidase II and TRH receptors in cerebellum is unclear at present time. One possible interpretation is that while cerebellum contains high level of mRNA, the pyroglutamyl peptidase II is synthesized and transported to the neuronal projections in the other areas of CNS.

In addition to the central nervous system, TRH was also identified in many peripheral organs, including gastrointestinal tract and pancreas, reproductive system, kidney, liver, lung, adrenals and body fluids (Prasad, 1987). One must view these earlier studies on the distribution of TRH with caution, since it is difficult to rule out the possible cross reactivity of some of the newly discovered TRH-like peptides (Khan et al., 1992) with the antibody against TRH. The presence of TRH receptors and its mRNA in peripheral organs supports a potential physiological function of TRH or related peptides in these organs (Fukusumi et al., 1995) although this role is far from clear. The relatively abundant levels of pyroglutamyl peptidase II enzymatic activity and mRNA in lung is of particular interest and requires further investigation.

Our studies also confirm the report of Schomburg and Bauer (Schomburg and Bauer, 1995) on the stimulating effect of T3 on pituitary pyroglutamyl peptidase II mRNA. This is also consistent with well defined studies on enzyme activity. In addition, we observed an increase of pyroglutamyl peptidase II mRNA in rat liver following treatment with T3. This is also consistent with the report that T3 increases liver pyroglutamyl peptidase II enzymatic activity (Scharfman et al., 1990). It is well established that T3

treatment increases the serum level of “thyroliberinase” in rats (White et al., 1976). The source of the serum enzyme is unknown, but in view of the effect of T3 on pyroglutamyl peptidase II activity and its mRNA, it is most likely derived from liver.

We did not observe any significant change in pyroglutamyl peptidase II mRNA by T3 in other peripheral organs or brain regions. Pyroglutamyl peptidase II appears to be encoded by only one gene (Schauder et al., 1994). The different responsiveness to T3 might be due to tissue specific transcription factors. An increase of pyroglutamyl peptidase II in frontal cortex following either a single injection of T3 or once daily injections for 10 or 14 days was reported (Suen and Wilk, 1990). As our present observation did not detect any change of the mRNA in the same region, other mechanisms acting at the post-transcriptional level might account for the elevation in enzymatic activity. Alternatively *in situ* hybridization might detect increases in mRNA in specific regions of the frontal cortex. Clinically TRH has been used diagnostically and also has been proposed to be of therapeutic value for its neurotropic, anti-trauma and anti-ataxia effects among others. Therefore it was of interest to determine whether TRH treatment could alter the levels of pyroglutamyl peptidase II mRNA *in vivo*. The dose we used simulated the doses generally used in *in vivo* studies. There has been *in vitro* evidence that pyroglutamyl peptidase II is regulated by TRH. In rat adenohypophyseal cell primary cultures, TRH decreases pyroglutamyl peptidase II activity (Vargas et al., 1994). In human Y-79 retinoblastoma cells, pyroglutamyl peptidase II activity is profoundly down-regulated by phorbol ester (Suen and Wilk, 1989). Since TRH receptors are linked to the activation of protein kinase C (Gershengom, 1986), the Y-79 cell studies indirectly support a role for TRH in the regulation of pyroglutamyl peptidase II. We did not detect any significant effect of TRH on

pyroglutamyl peptidase II mRNA levels in major rat organs or in any brain region. However the limited access of TRH to the CNS and its complex pharmacokinetics precludes a more definitive interpretation of these results.

Figure legends

Figure 1:

- A) Schematic representation of the pyroglutamyl peptidase II subclone for synthesis of riboprobe and standards. The clone contains an insert of the pyroglutamyl peptidase II cDNA sequence (Schauder et al., 1994) from nucleotides 219 to 702 within the T/A PCR II(tm) cloning vector (Invitrogen). The insert is flanked with a T7 RNA polymerase promotor site for synthesis of antisense RNA, and a SP6 RNA polymerase promotor site in the opposite direction for synthesis of sense RNA.
- B) Schematic diagram for synthesis of the antisense RNA probe. The clone is restricted with Hinc II inside the insert. The antisense RNA probe contains 209 bps of pyroglutamyl peptidase II cDNA sequence and 68 bps of plasmid vector sequence.
- C) Schematic diagram for synthesis of the sense RNA probe. The plasmid is linearized by treatment with Xho I.

Figure 2:

Relationship between volumes of hybridized bands and pg in vitro synthesized sense RNA as determined by a ribonuclease protection assay.

- A) Autoradiograph of increasing quantities of RNA (+) standards. Conditions as described in Methods. Lane 1, 16 pg; Lane 2, 8 pg; lane 3, 6 pg; lane 4, 4 pg; lane 5, 2 pg; lane 6, 1 pg; lane 7, 0.5 pg; lane 8, represents the negative control containing total yeast RNA.

- B) Standard curve for the quantitation of pyroglutamyl peptidase II mRNA. There is an excellent linear correlation between increasing quantities of pyroglutamyl peptidase II standard sense RNA and the volume of hybrids as

quantitated by a computer assisted phosphoimager system ($r>0.99$ in all experiments).

Figure 3:

Pyroglutamyl peptidase II mRNA in rat organs determined by a ribonuclease protection assay as described in Methods. Each lane contained 80 μg total RNA. Lane 1, liver; lane 2, lung; lane 3, heart; lane 4, spleen; lane 5, intestine; lane 6, muscle; lane 7, testis; lane 8, duodenum/pancreas; lane 9, kidney; lane 10, DNA markers. Amounts calculated as fg/ μg total RNA are presented in Table 1.

Figure 4 :

Quantitation of pyroglutamyl peptidase II mRNA in rat brain regions, pituitary and spinal cord by a ribonuclease protection assay as described in Methods. A) Correlation of the hybrid volumes with increasing amounts of brain total RNA. There is excellent linear correlation between the quantity of brain total cytosolic RNA and the volume of the hybrids with the radioprobe as determined by a computer assisted phosphoimager system ($r>0.99$).

B) Pyroglutamyl peptidase II mRNA in rat brain regions, pituitary and spinal cord. Values in parenthesis indicate amount of total RNA (μg) applied for analysis. Representative cases are shown. Lane 1, amygdala (17); lane 2, hippocampus (25); lane 3, thalamus (20); lane 4, hypothalamus (20); lane 5, olfactory bulb (20); lane 6, posterior cortex (14); lane 7, frontal cortex (26); lane 8, pituitary (83); lane 9, spinal cord (20); lane 10, brainstem (20); lane 11, cerebellum (38); lane 12, striatum (8). Amounts calculated as fg/ μg total RNA are presented in Table 2.

Figure 5:

Effect of T3 and TRH on pyroglutamyl peptidase II mRNA in rat pituitary and frontal cortex. Rats received a single subcutaneous injection of 100 µg T3, or a single intravenous injection of 300 µg TRH, or vehicle only. Individual pituitaries and frontal cortices were removed 16 h after treatment and total cytosolic RNA extracted. The RNA was quantitated by absorption at 260 nm. Solution hybridization ribonuclease assay was performed as described in Methods. Lanes 1-3 pituitary: lane 1, control; lane 2, T3; lane 3 TRH. Lanes 4-6 frontal cortex: lane 4, control; lane 5, T3, lane 6, TRH. Values reported in Tables 3 and 4.

Figure 6:

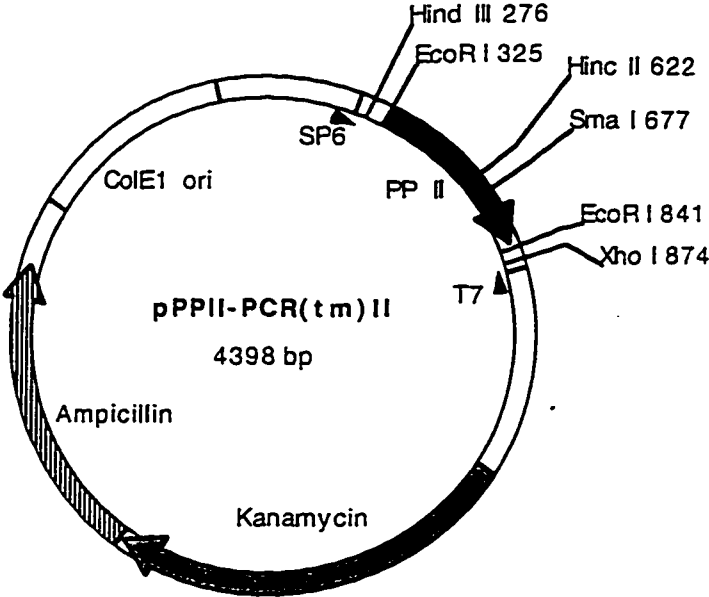
Effect of T3 and TRH on pyroglutamyl peptidase II mRNA in rat liver and lung. Rats received a single subcutaneous injection of 100 µg T3, or a single intravenous injection of 300 µg TRH, or vehicle only. Liver and lung samples were removed 16 h after treatment and total cytosolic RNA extracted. Solution hybridization ribonuclease assay was performed as described in Methods.

A) Liver: Lane 1, control; lane 2, T3, lane 3, TRH.

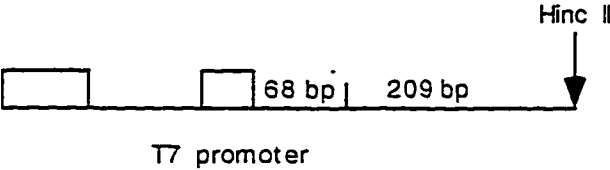
B) Lung: Lane 1, control; lane 2, T3, lane 3, TRH.

Figure 1

A



B



C

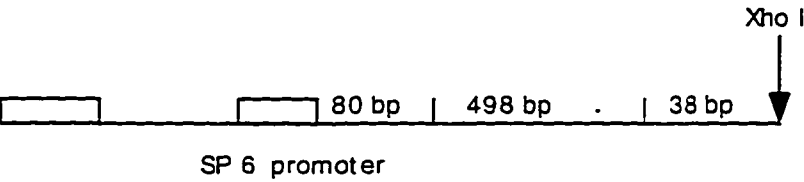


Figure 2

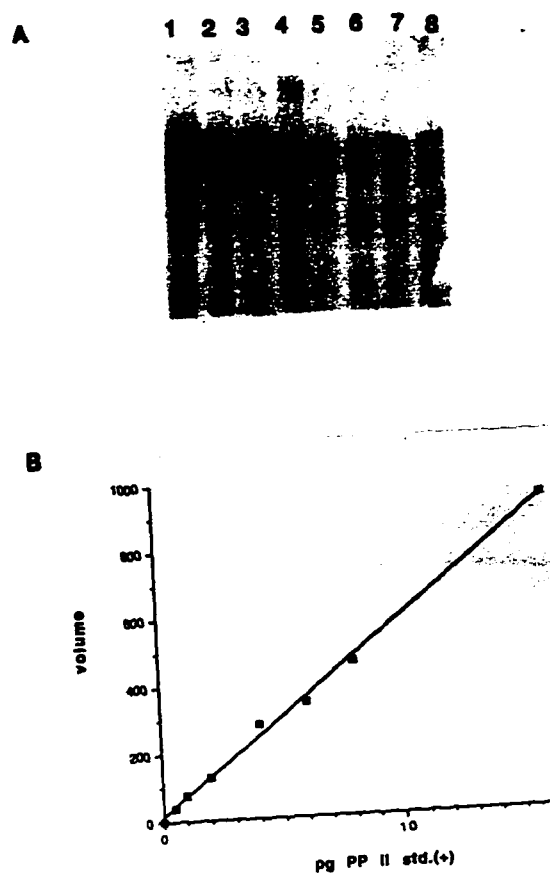


Figure 3

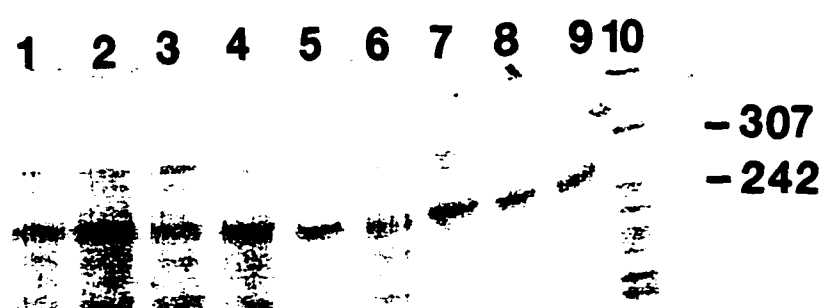


Figure 4

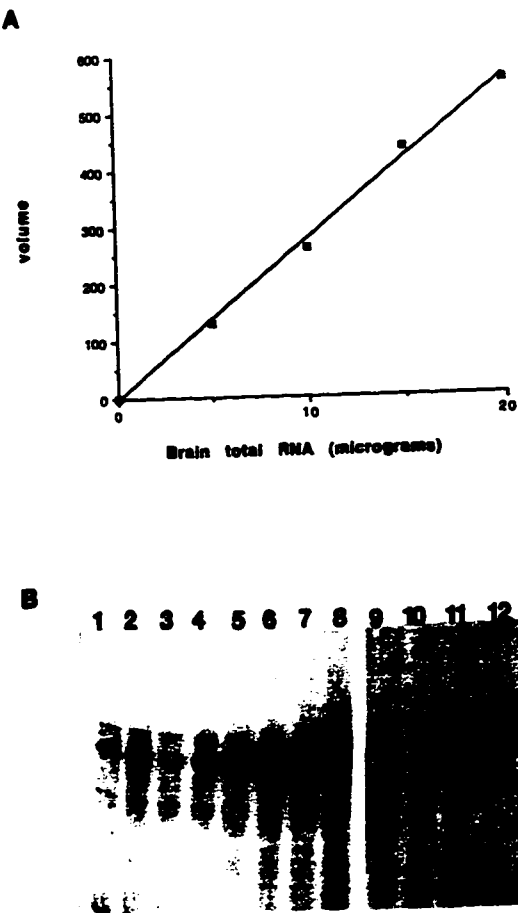


Figure 5



Figure 6

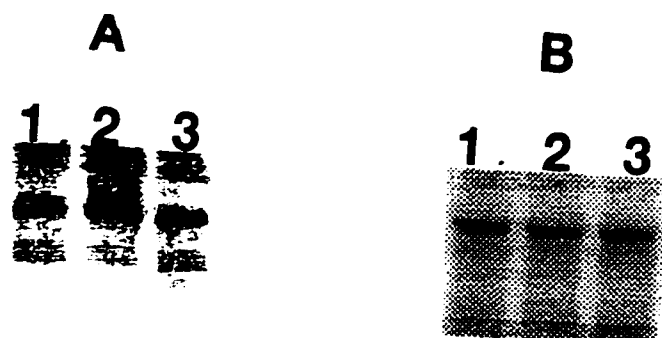


Table 1. Pyroglutamyl peptidase II mRNA levels and enzymatic activity in rat organs.

Organ	mRNA (fg/ μ g total RNA)	Specific Activity (units/mg protein)
Brain	705 $\pm$ 47	4.8 $\pm$ 0.6
Duodenum/Pancreas	21 $\pm$ 2	0.05 $\pm$ 0.02
Heart	22 $\pm$ 2	0.04 $\pm$ 0.01
Intestine	21 $\pm$ 3	0.06 $\pm$ 0.02
Kidney	29 $\pm$ 2	0.03 $\pm$ 0.01
Pituitary	42 $\pm$ 2*	0.2 $\pm$ 0.1
Liver	13 $\pm$ 0.7	0.1 $\pm$ 0.1
Lung	116 $\pm$ 13	1.2 $\pm$ 0.3
Muscle	27 $\pm$ 3	0.03 $\pm$ 0.01
Spleen	31 $\pm$ 3	0.1 $\pm$ 0.03
Testis	30 $\pm$ 2	0.03 $\pm$ 0.01

Pyroglutamyl peptidase II mRNA levels in rat organs were determined by a solution hybridization ribonuclease protection assay as described in Materials and Methods. Values represent the mean $\pm$ S.E., n=6 for each group.

Pyroglutamyl peptidase II specific activity was determined as described in Materials and Methods. A unit of enzyme activity is defined as the release of 1 nmol naphthylamine (NA) per hour from the substrate pGlu-His-Pro-NA. Data are mean values of three samples $\pm$ SEM.

* significantly greater than duodenum/pancreas, heart, intestine, kidney, muscle, and testes $p < 0.01$; and significantly greater than spleen $p < 0.05$ (Student's T-test).

Table 2 Pyroglutamyl peptidase II mRNA levels and enzymatic activity in rat brain regions and spinal cord.

<u>Region</u>	mRNA (fg/ μ g total RNA)	Specific Activity (units/mg protein)
Amygdala	418 $\pm$ 28	3.1 $\pm$ 0.3
Brainstem	129 $\pm$ 19	0.6 $\pm$ 0.2
Cerebellum	904 $\pm$ 97	2.3 $\pm$ 0.3
Frontal Cortex	802 $\pm$ 49	7.5 $\pm$ 0.5
Hippocampus	929 $\pm$ 67	7.7 $\pm$ 0.8
Hypothalamus	229 $\pm$ 29	2.8 $\pm$ 0.2
Olfactory Bulb	628 $\pm$ 55	7.0 $\pm$ 0.4
Posterior Cortex	1056 $\pm$ 83	8.1 $\pm$ 0.6
Spinal Cord	219 $\pm$ 26	1.6 $\pm$ 0.2
Striatum	394 $\pm$ 47	1.6 $\pm$ 0.2
Thalamus	280 $\pm$ 19	2.8 $\pm$ 0.2

Pyroglutamyl peptidase II mRNA levels in rat brain regions were determined by a solution hybridization ribonuclease protection assay as described in Materials and Methods. Values represent mean $\pm$ S.E, n=6 for each group.

Pyroglutamyl peptidase II activity was determined as described in Materials and Methods. A unit of enzyme activity is defined as the release of 1 nmol NA per hour from the substrate pGlu-His-Pro-NA. Data are mean values of three samples $\pm$ SEM.

Table 3. Effect of T3 and TRH on pyroglutamyl peptidase II mRNA concentrations in rat organs .

<u>Organ</u>	<u>mRNA (fg/μg total RNA)</u>		
	Control	T3	TRH
Brain	740 $\pm$ 66	736 $\pm$ 64	724 $\pm$ 51
Duodenum/Pancreas	19 $\pm$ 2	20 $\pm$ 2	21 $\pm$ 1
Heart	22 $\pm$ 3	18 $\pm$ 2	19 $\pm$ 3
Intestine	22 $\pm$ 2	22 $\pm$ 3	19 $\pm$ 1
Kidney	29 $\pm$ 2	28 $\pm$ 3	29 $\pm$ 3
Liver	14 $\pm$ 1	50 $\pm$ 18 *	15 $\pm$ 3
Lung	116 $\pm$ 15	125 $\pm$ 17	115 $\pm$ 15
Muscle	27 $\pm$ 5	28 $\pm$ 2	23 $\pm$ 1
Pituitary	41 $\pm$ 4	169 $\pm$ 16**	35 $\pm$ 3
Spleen	34 $\pm$ 5	32 $\pm$ 5	33 $\pm$ 5
Testis	31 $\pm$ 2	30 $\pm$ 2	29 $\pm$ 3

Male Sprague Dawley rats were treated with a single dose of either 100 μ g T3 subcutaneously or 300 μ g TRH intravenously and sacrificed 16 h later. Pyroglutamyl peptidase II mRNA levels were quantitated by a solution hybridization ribonuclease protection assay as described in Materials and Methods. Values represent mean $\pm$ S.E., n=4 for each group, with the exception of the liver where n=8.

** p<0.01; * p<0.05

Table 4. Effect of T3 and TRH on pyroglutamyl peptidase II mRNA levels in rat brain regions and spinal cord

Region	mRNA (fg/ μ g total RNA)		
	Control	T3	TRH
Amygdala	427 $\pm$ 38	421 $\pm$ 23	391 $\pm$ 49
Brainstem	133 $\pm$ 21	132 $\pm$ 19	124 $\pm$ 14
Cerebellum	909 $\pm$ 36	921 $\pm$ 65	959 $\pm$ 98
Frontal Cortex	793 $\pm$ 51	811 $\pm$ 62	767 $\pm$ 23
Hippocampus	941 $\pm$ 61	948 $\pm$ 53	956 $\pm$ 61
Hypothalamus	230 $\pm$ 48	242 $\pm$ 49	223 $\pm$ 26
Olfactory Bulb	584 $\pm$ 63	639 $\pm$ 62	599 $\pm$ 53
Posterior Cortex	1078 $\pm$ 80	1003 $\pm$ 80	1028 $\pm$ 77
Spinal Cord	226 $\pm$ 28	260 $\pm$ 42	217 $\pm$ 20
Striatum	399 $\pm$ 14	406 $\pm$ 72	352 $\pm$ 33
Thalamus	284 $\pm$ 23	282 $\pm$ 18	280 $\pm$ 27

Treatment as described in legend to Table 3. Values expressed as mean $\pm$ S.E., n= 4 for each group.

Table 5. Effect of T3 and TRH on pyroglutamyl peptidase II activities in rat organs .

<u>Organ</u>	Specific Activity (units/mg protein)		
	Control	T3	TRH
Brain	4.81± 0.57	4.83 ± 0.61	4.79 ± 0.61
Duodenum/Pancreas	0.05 ± 0.02	0.05 ± 0.02	0.05 ± 0.02
Heart	0.04 ± 0.02	0.04 ± 0.01	0.04 ± 0.02
Intestine	0.06 ± 0.02	0.06 ± 0.02	0.06 ± 0.02
Kidney	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
Liver	0.11 ± 0.1	0.20 ± 0.11 *	0.10 ± 0.1
Lung	1.21 ± 0.41	1.22 ± 0.42	1.20 ± 0.38
Muscle	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
Pituitary	0.20 ± 0.07	1.13 ± 0.11**	0.19 ± 0.09
Spleen	0.11 ± 0.02	0.12 ± 0.02	0.10 ± 0.02
Testis	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01

Male Sprague Dawley rats were treated with a single dose of either 100 µg T3 subcutaneously or 300 µg TRH intravenously and sacrificed 16 h later.

Pyroglutamyl peptidase II specific activity was determined as described in Materials and Methods. A unit of enzyme activity is defined as the release of 1 nmol naphthylamine (NA) per hour from the substrate pGlu-His-Pro-NA. Data are mean values of three samples ± SEM. Values represent mean ± S.E., n=4 for each group, with the exception of the liver where n=8.

** p<0.01; * p<0.05

Table 6. Effect of T3 and TRH on pyroglutamyl peptidase II activities in rat brain regions and spinal cord

Region	Specific Activity (units/mg protein)		
	Control	T3	TRH
Amygdala	3.1 ± 0.3	3.1 ± 0.3	3.1 ± 0.3
Brainstem	0.61 ± 0.2	0.64 ± 0.2	0.62 ± 0.2
Cerebellum	2.4 ± 0.4	2.3 ± 0.4	2.3 ± 0.4
Frontal Cortex	7.5 ± 0.5	7.5 ± 0.5	7.5 ± 0.5
Hippocampus	7.7 ± 0.8	7.7 ± 0.8	7.7 ± 0.8
Hypothalamus	2.8 ± 0.2	2.8 ± 0.2	2.8 ± 0.2
Olfactory Bulb	7.0 ± 0.4	7.0 ± 0.5	7.0 ± 0.5
Posterior Cortex	8.1 ± 0.6	8.0 ± 0.7	8.1 ± 0.7
Spinal Cord	1.6 ± 0.2	1.6 ± 0.2	1.6 ± 0.2
Striatum	1.6 ± 0.2	1.6 ± 0.2	1.6 ± 0.2
Thalamus	2.8 ± 0.3	2.8 ± 0.2	2.8 ± 0.3

Treatment as described in legend to Table 3. Pyroglutamyl peptidase II activity was determined as described in Materials and Methods. A unit of enzyme activity is defined as the release of 1 nmol NA per hour from the substrate pGlu-His-Pro-NA. Values expressed as mean ± S.E., n= 3 for each group.

Chapter 4

Regulation of pyroglutamyl peptidase II mRNA in GH3 cells

Abstract

The potential regulatory effects of TRH, thyroid hormone and epidermal growth factor (EGF) on the steady state levels of the mRNA of the specific TRH-degrading enzyme pyroglutamyl peptidase II were investigated in GH3 cells. Pyroglutamyl peptidase II mRNA was measured by a quantitative solution hybridization ribonuclease assay. TRH decreased pyroglutamyl peptidase II mRNA. A maximal effect was observed 16 h after exposure to 10^{-7} M TRH. The protein kinase C (PKC) activators phorbol-12 β -myristate-13-acetate (PMA), and phorbol-12,13-dibutyrate also decreased pyroglutamyl peptidase II mRNA levels, whereas the inactive phorbol ester analog 4- α -TPA had no effect. The PKC inhibitor H-7 inhibited the increase in pyroglutamyl peptidase II mRNA produced by TRH and PMA. Exposure to T3 at concentrations of 10^{-10} M to 10^{-6} M, for time periods of 2 h to 24 h did not change pyroglutamyl peptidase II mRNA levels. EGF treatment (5×10^{-9} M for 5 days) produced a small (30 %) but significant increase in pyroglutamyl peptidase II mRNA. The down-regulation of pyroglutamyl peptidase II mRNA by TRH in GH3 cells agrees with earlier studies on adenohypophyseal cultures (Vargas, et al., *Neuroendocrinol.* 60: 323-340 (1994)) and may provide a positive feedback mechanism for the action of TRH on pituitary lactotropes.

TRH was first isolated as a hypothalamic hypophysiotropic hormone which promoted the secretion of pituitary thyroid-stimulating hormone (TSH) (1). In addition to its action on TSH, TRH was shown to stimulate the release of prolactin (2) with a similar potency (3). Under certain pathological conditions, TRH can also stimulate the release of other pituitary hormones such as growth hormone and ACTH (4).

The actions of TRH on pituitary thyrotropes and lactotropes are mediated by high affinity, specific receptors (5 -7). The TRH receptor has been cloned and shown to belong to the large family of G protein-coupled receptors (GPCRs) (8). The interaction of TRH and its receptor is mediated via G proteins coupled by the phospholipase C activation pathway (9,10).

A highly specific and efficient degrading and inactivating mechanism for TRH in the pituitary cell membrane is provided by the ectoenzyme pyroglutamyl peptidase II (11). Several studies have shown that the availability of TRH to pituitary cells may in part be regulated by this inactivating enzyme. Thus the activity of pyroglutamyl peptidase II in pituitary (12) and serum (13) is up-regulated by thyroid hormone *in vivo*. The activity of this enzyme is also down-regulated in primary pituitary cultures by TRH (14).

It has been proposed that in the pituitary, pyroglutamyl peptidase II is preferentially located on lactotrope cells (15), although the cell type responding to the regulation by thyroid hormone and TRH has not been identified. The GH3 cell is a well characterized prolactin and growth hormone secreting rat pituitary tumor cell line. It contains functional TRH receptors (16) as well as low but measurable levels of pyroglutamyl peptidase II (17). We sought to determine whether the GH3 cell could be used to study pyroglutamyl peptidase II regulation. Epidermal growth factor (EGF) has been shown to remarkably increase the population of prolactin-secreting cell types in GH3 cultures (18). If

indeed pyroglutamyl peptidase II is located preferentially on prolactin secreting cells, the shift to a prolactin secreting cell population may result in an increase in this enzyme. We have established a highly sensitive and quantitative solution hybridization ribonuclease protection assay to detect the mRNA level of pyroglutamyl peptidase II in GH3 cells (Lin et al., submitted). We report here the effects of treatment of GH3 cells with TRH, thyroid hormone and EGF on pyroglutamyl peptidase II mRNA levels.

Materials and Methods

Materials

GH3 cells were from American Type Cell Collection (ATCC), Rockville, MD. T3, EGF, TRH, phorbol 12 β -myristate-13-acetate (PMA), phorbol 12,13-dibutyrate, 4 α -TPA and 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine dihydrochloride (H7) were purchased from Sigma Chemical Co., St. Louis, MO. Cell culture media, Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum, penicillin (10,000 U/ml) streptomycin (10,000 μ g/ml) and trypsin/EDTA were from Gibco, Grand Island, NY.

Cell culture

GH3 cells were grown in monolayer culture at 37 °C in a humidified atmosphere of 5% carbon dioxide and 95% air. Cell culture media consisted of DMEM, 15% fetal bovine serum, 100 U penicillin and 100 μ g/ml streptomycin. Cultures were maintained in 175 cm² flasks. Cells for experiments were plated in 25 cm² plates by treating the cells with 0.05% trypsin/EDTA for 2-3 min and harvesting at a 3 fold lower density. Experiments were conducted at near confluence by replacing the fresh medium with media containing substances to be tested. The cells were washed three times with DMEM before being subjected to total RNA isolation.

Isolation of total cytoplasmic RNA

Total cytoplasmic RNA was isolated from GH3 cells by the method of Lazar and Blum (19). Briefly, GH3 cells were treated with ice-cold lysis buffer (10 mM Tris-HCl, pH 7.4, 1.5 mM MgCl₂, 0.5% Nonidet P-40, 0.25% sodium deoxycholate, 0.3 M sucrose). Lysates were layered over a sucrose cushion (10 mM Tris-HCl,

pH 7.4, 1.5 mM MgCl₂, 0.4 M sucrose) and centrifuged at 5000 g for 10 min and at 4^o C. The upper phase (600 µl) was transferred to a new tube and 60 µl 10X SET (10% SDS, 50 mM EDTA, 100 mM Tris-HCl pH 8.0) plus 6 µl proteinase K (10 mg/ml) was added. After incubation at 45^o C for 1 h, 66 µl 3M NH₄Ac, 300 µl phenol and 300 µl chloroform were added and the tubes were vortexed and centrifuged. The aqueous phase was transferred to new tubes, 730 µl of cold isopropanol added and the tubes stored at -20^o C overnight. The RNA was recovered by centrifuging at 16 000 g for 10 min. The RNA pellets were washed with 70% ethanol, dried, and resuspended in diethylpyrocarbonate-treated deionized water. RNA concentration was determined by A₂₆₀. The integrity of the RNA was examined by agarose gel electrophoresis in the presence of ethidium bromide. The chemicals and solutions used in the experiments with RNA were tested as RNase free.

Synthesis of ³²P-labelled antisense riboprobe and unlabelled RNA standards.

The synthesis of the ³²P-labelled riboprobe and unlabelled RNA standards was conducted as previously described (Lin et al., submitted). Briefly a cDNA sequence of 483 nucleotides of pyroglutamyl peptidase II (20), from nucleotides 287 to 784 was amplified by PCR. A major PCR product was a band slightly smaller than 500 bps. It was ligated to a TA cloning plasmid vector PCRTM II flanked by T7 and SP6 RNA polymerase. The plasmid was enzymatically restricted 5' within the insert with Hinc II to generate an antisense riboprobe of 277 bps, containing 209 bps of the pyroglutamyl peptidase II cDNA fragment and 68 bps of plasmid DNA. The sense-strand RNA standard was prepared by enzymatically restricting the plasmid 3' within the multiple cloning site with Xho I. The linearized plasmid DNAs were purified by agarose gel electrophoresis and recovered by the GeneClean DNA isolation technique (Bio

101). The synthesis of the ^{32}P -labelled antisense riboprobe of 277 bps was performed with the aid of a Gemini kit. The *in vitro* transcription occurred after the addition of 0.5 μl RNA polymerase T7 (5U/ml). The synthesized riboprobe was purified by chromatography on a Sephadex G 100 column. The quality of the probe was examined by acrylamide gel electrophoresis. An RNA standard was synthesized and purified in a similar way. In this case, the *in vitro* transcription occurred after the addition of RNA polymerase SP₆ (25 U/ml). The amount of RNA standard was calculated, aliquoted and stored at -80° C.

Solution hybridization ribonuclear protection assay.

The solution hybridization ribonuclear protection assay was conducted as previously described (Lin et al., submitted). RNA standards consisted of increasing quantities of *in vitro* transcribed sense-stand RNA, generally from 0 pg to 16 pg. Total cytosolic RNA from a single GH3 cell plate ranged from 60 μg to 80 μg . RNA standards and RNA samples were dried down in a vacuum speed dryer and resuspended in 30 μl hybridization buffer containing 0.5 -1 μl freshly synthesized riboprobe (100-200 pg). Samples were heated at 85° C for 5 min, and then incubated at 45° C overnight (16h). RNA:RNA hybrid pellets were recovered by centrifugation, washed with 70% ethanol, resuspended in 8 μl gel loading buffer, and analyzed on 4% non-denaturing acrylamide gels. The gels were dried, and placed on a phosphorimager screen (Molecular Dynamics). The volumes of hybridized bands analyzed by a computer assisted phosphorimager system were quantitated. The standard curve and the sample amounts were calculated with the aid of computer software (Statview). Because of the size difference between the hybrids with synthesized standard (+) RNA and tissue isolated mRNA, a correction factor, 277/209, was applied to the calculation of sample pyroglutamyl peptidase II mRNA.

Results

Detection of pyroglutamyl peptidase II mRNA in GH3 cells by a solution hybridization ribonuclease protection assay

Pyroglutamyl peptidase II mRNA was detected by a sensitive and quantitative solution hybridization ribonuclease protection assay. Total yeast RNA was unable to protect the riboprobe from digestion by RNase A and T1 (lane zero, Fig 1A). The volume of hybrids as quantitated by a computer assisted phosphoimager system was highly correlated to increasing quantities of pyroglutamyl peptidase II standard sense RNA ($r > 0.98$ in all experiments) (Fig. 1B). There was also an excellent linear correlation between the increasing quantity of GH3 cell total cytosolic RNA and the volume of hybrids (Fig. 1C).

TRH down-regulates pyroglutamyl peptidase II mRNA in GH3 cells;

TRH decreased the level of pyroglutamyl peptidase II mRNA in GH3 cells. This effect was both time and dose-dependent. A maximal effect occurred 16 h after exposure to 10^{-7} M TRH and a significant decrease was observed as early as 2 h after exposure to 10^{-7} M TRH (Fig 2). Significant changes occurred at TRH concentrations of 10^{-10} M to 10^{-6} M (Fig. 3). Pyroglutamyl peptidase II activity was downregulated by TRH (Table 1).

Phorbol esters down-regulate pyroglutamyl peptidase II mRNA in GH3 cells

Involvement of PKC in the regulation of PP II mRNA levels was demonstrated by the effect of direct PKC activators. PMA decreased pyroglutamyl peptidase II mRNA in GH3 cells in a dose and time-dependent manner (Figs. 4,5). The active phorbol ester analog phorbol-12,13-dibutyrate had a similar effect to PMA, but the inactive analog 4- α -TPA had no effect on

pyroglutamyl peptidase II mRNA levels. The protein kinase C inhibitor H-7 was able to inhibit the effects of both TRH and PMA on pyroglutamyl peptidase II mRNA and enzyme activity (Table 1).

Effect of thyroid hormone on pyroglutamyl peptidase II mRNA in GH3 cells

T3 at concentrations of 10^{-10} M to 10^{-6} M and at times of exposure from 0.5 h to 24 h failed to alter pyroglutamyl peptidase II mRNA levels or enzyme activity in GH3 cells. Similarly there was no significant difference when T3 was added to culture media which was stripped of thyroid hormone (Table 2).

Effect of EGF on pyroglutamyl peptidase II mRNA in GH3 cells

GH3 cells were treated with three concentrations of EGF (0.1 nM, 1nM, and 5nM) for 3 days, 5 days and 7 days. Media was replaced each day with fresh media containing EGF. A significant increase in pyroglutamyl peptidase II mRNA in GH3 cells was found with all doses of EGF at each of the days of incubation. A maximal effect (30% increase) occurred following treatment with 5nM EGF for 5 days (Fig. 6). Specific activity of pyroglutamyl peptidase II was also increased from 0.11 ± 0.02 to 0.15 ± 0.02 (unit/mg) with 5nm EGF for 5 days also observed ($n=3$, $p<0.05$).

Discussion

These studies provide evidence that pyroglutamyl peptidase II gene expression can be down-regulated by TRH. The involvement of PKC activation in this process is shown by the results with active and inactive phorbol ester analogs, and the inhibition by the PKC inhibitor H7. Our results are consistent with previous findings from this laboratory in human Y-79 retinoblastoma cells which have shown that pyroglutamyl peptidase II activity is bi-phasically down-regulated by phorbol ester. In these studies it was proposed that the rapid inactivation seen from 5-15 min after treatment with phorbol ester was due to a direct phosphorylation of the enzyme (21), whereas the secondary decrease seen after 2-6 h exposure to phorbol ester was due to a decrease in enzyme synthesis (22). Recently it was reported that TRH decreased pyroglutamyl peptidase II activity in primary cultures of rat adenohypophyseal cells by a PKC-mediated mechanism with a significant effect seen after 3 h (14). It thus appears that the down-regulation of pyroglutamyl peptidase II activity by TRH in adenohypophyseal cells, is mediated at least in part through a decrease in the steady state mRNA levels. In other studies we have been unable to detect any effect of TRH *in vivo* on pituitary pyroglutamyl peptidase II mRNA (Lin et al., submitted). However the limited access of TRH to the CNS and its complex pharmacokinetics precludes a more definitive interpretation of these results.

The involvement of PKC is in agreement with evidence that the interaction of TRH with its receptor is mediated via G proteins coupled to the phospholipase C pathway (9,10). Binding of TRH to its receptor results in the stimulation of phosphoinositide turnover with an increase in cellular levels of two messengers, diacylglycerol and IP3. IP3 activates PKC while diacylglycerol

increases the cytosolic Ca^{2+} level, which in turn activates Ca^{2+} calmodulin-dependent protein kinase (16, 23,24). The role of the Ca^{2+} dependent protein kinase in the regulatory effects of TRH on pyroglutamyl peptidase II if any, remains to be evaluated. The down-regulatory effects of TRH on its degrading enzyme may provide a positive feedback mechanism to insure the availability and duration of TRH on pituitary target cells. Since the GH3 cell is a prolactin-secreting cell line, it is likely that the adenohypophyseal cell type responding to TRH is the lactotrope.

The stimulatory effects of T3 on pituitary pyroglutamyl peptidase II enzymatic activity and mRNA *in vivo* have been well documented (12, 20, 25; Lin, et al, submitted). Similarly thyroid hormone treatment down-regulates rat adenohypophyseal TRH receptor mRNA (25). This has led to the attractive hypothesis that the secretion of prolactin is under the reciprocal regulation of both TRH receptors and pyroglutamyl peptidase II (25). We have not detected any changes in pyroglutamyl peptidase II mRNA or enzyme activity in GH3 cells following treatment with thyroid hormone. Yamada et al., (26) reported that thyroid hormone did not significantly change TRH receptor mRNA in GH3 cells. Since thyroid hormone tightly and reciprocally regulates the mRNA levels of the TRH receptor and pyroglutamyl peptidase II in the rat adenohypophysis *in vivo*, but has no effect on these parameters in the GH3 cell, a fundamental difference in the *in vivo* and *in vitro* mechanisms regulating the TRH receptor and the TRH degrading enzyme is revealed.

GH3 cells consist of classical somatotropes and lactosomatotropes, while containing only about 0.5% of classical lactotropes. The GH3 cell thus differs from the mature rat pituitary which contains 50-90% classical lactotropes in all

prolactin secreting cells (27,28). Studies by Bauer et al., (15) have shown that pyroglutamyl peptidase II is localized primarily if not exclusively on lactotropes. An induction of classical lactotropes from about 0.5 % to 8 % in GH3 cells by EGF treatment was reported by Felix, et al (18). We therefore anticipated that treatment of GH3 cells with EGF would result in a dramatic increase in the mRNA of pyroglutamyl peptidase II. Although we observed a significant increase in pyroglutamyl peptidase II mRNA levels with 0.1nM, 1nM and 5nM EGF following treatment for 3 , 5 and 7 days, the magnitude of the effect was quite modest. However we did not determine the percentage of classical lactotropes before and after EGF treatment. EGF has been demonstrated to modulate the TRH response and the TRH receptor concentration in pituitary cells (29). It is therefore reasonable to propose that pyroglutamyl peptidase II is also regulated by EGF *in vivo*.

Figure Legends

Figure 1

Pyroglutamyl peptidase II mRNA in GH3 cells as determined by a ribonuclease protection assay.

A) Autoradiograph of increasing quantities of RNA (+) standards. Conditions as described in Methods. Lane 1 represents the negative control containing total yeast RNA. Lane 2, 0.25 pg; lane 3, 0.5 pg; lane 4, 1 pg; lane 5, 2 pg; lane 6, 4 pg; lane 7, 6 pg; lane 8, 8 pg.

B) Standard curve for the quantitation of pyroglutamyl peptidase II mRNA.

There is an excellent linear correlation between increasing quantities of pyroglutamyl peptidase II standard sense RNA and the volume of hybrids as quantitated by a computer assisted phosphoimager system ($r > 0.98$ in all experiments).

C) Autoradiograph of increasing quantities of GH3 cell total cytosolic RNA. Lane 1, 0 μ g; lane 2, 5 μ g; lane 3, 10 μ g; lane 4, 20 μ g; lane 5, 40 μ g; lane 6 as comparison, 15 μ g whole brain cytosolic RNA.

Figure 2:

Effect of TRH on pyroglutamyl peptidase II mRNA in GH3 cells as a function of the time of exposure. GH3 cells were exposed to 10^{-7} M TRH for periods of time from 0.5 h to 24 h. Total cytosolic RNA was isolated and pyroglutamyl peptidase II mRNA quantitated by a solution hybridization ribonuclease protection assay. Each data point represents the mean $\pm$ S.E. of 4 experiments. * $P < 0.05$ compared to control.

Figure 3:

Dose- response effect of TRH on pyroglutamyl peptidase II mRNA levels in GH3 cells. GH3 cells were exposed for 16 h to TRH at concentrations from 10^{-10} M to 10^{-6} M. Total cytosolic RNA was isolated and pyroglutamyl peptidase II mRNA quantitated by a solution hybridization ribonuclease protection assay. Each data point represents the mean $\pm$ S.E. of 4 experiments. * $P < 0.05$ compared to control.

Figure 4

Effect of PMA treatment on pyroglutamyl peptidase II mRNA levels in GH3 cells as a function of time of exposure. GH3 cells were treated with 10^{-7} M PMA for periods of time from 0.5 h to 24 h. Total cytosolic RNA was isolated and pyroglutamyl peptidase II mRNA quantitated by a solution hybridization ribonuclease protection assay. Each data point is the mean $\pm$ S.E. of 4 experiments. * $P < 0.05$ compared to control.

Figure 5:

Dose-response effect of PMA on pyroglutamyl peptidase II mRNA levels in GH3 cells. GH3 cells were exposed to PMA at concentrations from 10^{-10} M to 10^{-6} M for 16 h. Total cytosolic RNA was isolated and pyroglutamyl peptidase II mRNA quantitated by a solution hybridization ribonuclease protection assay. Each data point is the mean $\pm$ S.E. of 4 experiments. * $P < 0.05$ compared to control.

Figure 6:

Effect of EGF on pyroglutamyl peptidase II mRNA levels in GH3 cells. GH3 cells were exposed to EGF at concentrations of 0.1 nM, 1nM, and 5nM for

3, 5 and 7 days. Media containing EGF were replaced each day. Total cytosolic RNA was isolated and pyroglutamyl peptidase II mRNA measured by a solution hybridization ribonuclease protection assay. Data represents the mean $\pm$ S.E. (n=4). * $P < 0.05$ compared to control.

Figure 1

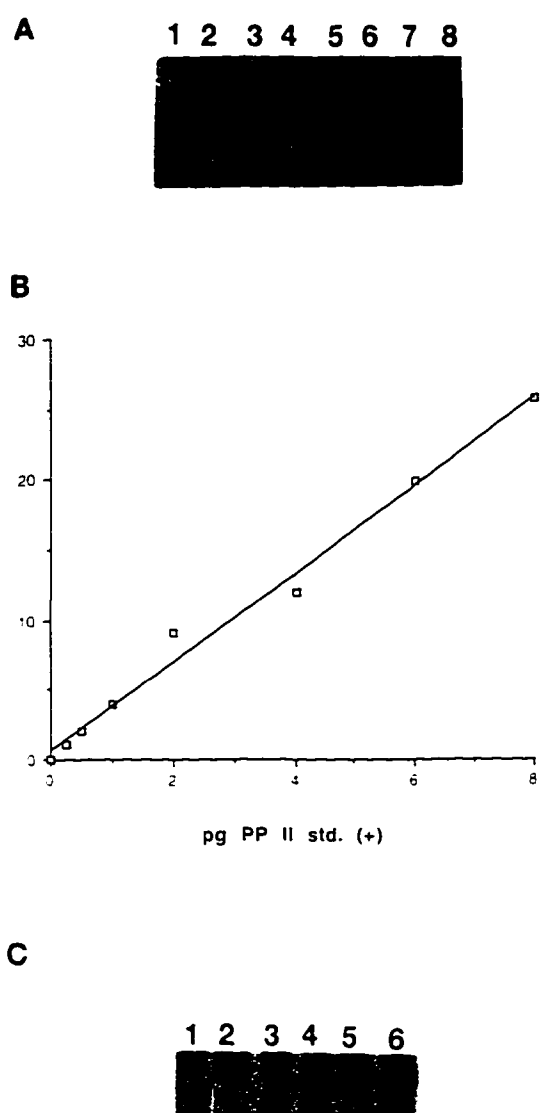


Figure 2

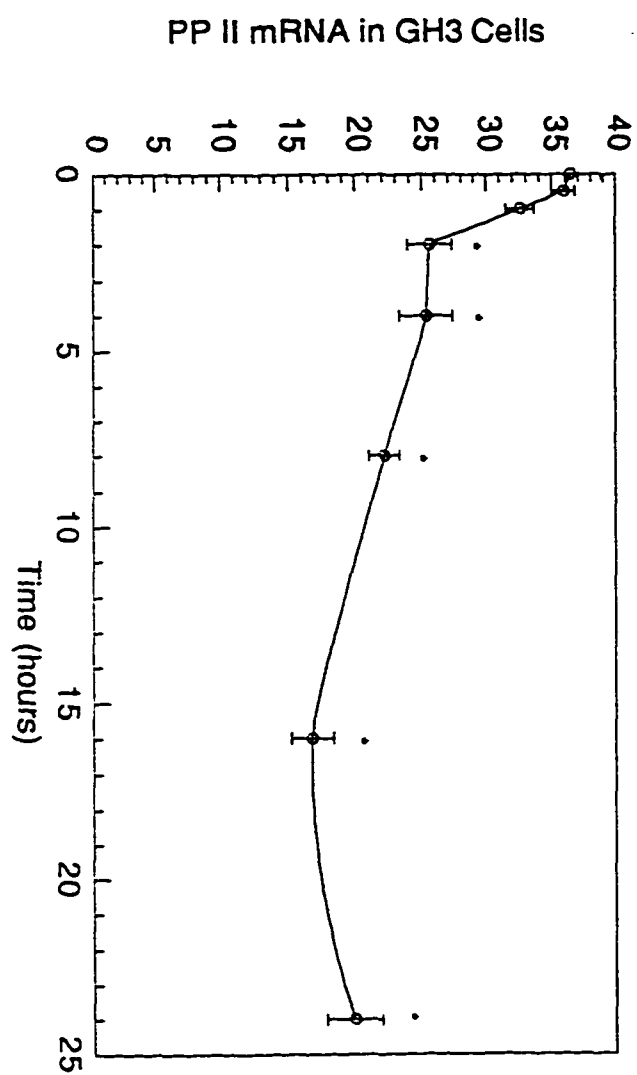


Figure 3

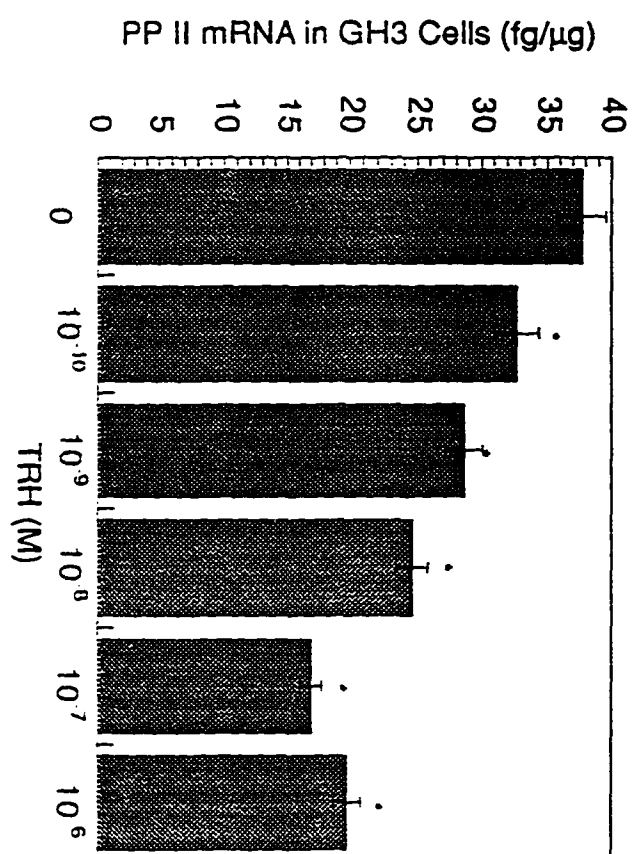


Figure 4

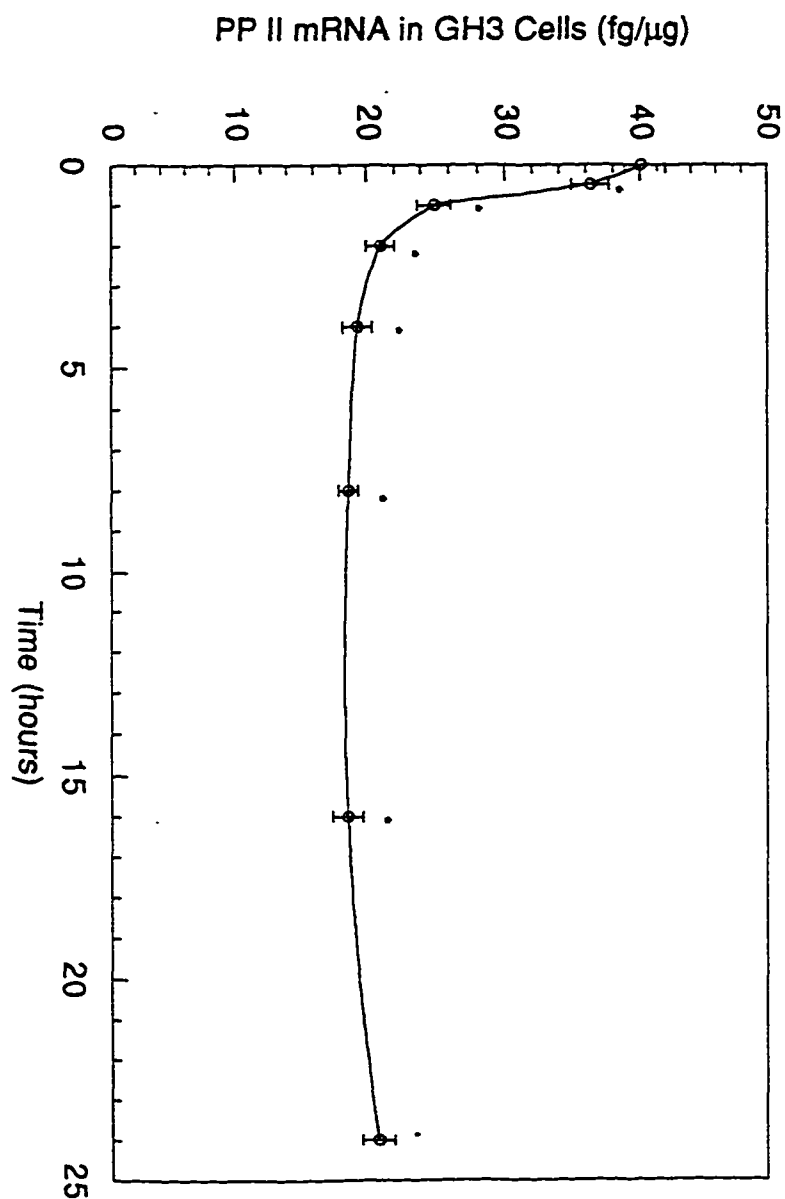


Figure 5

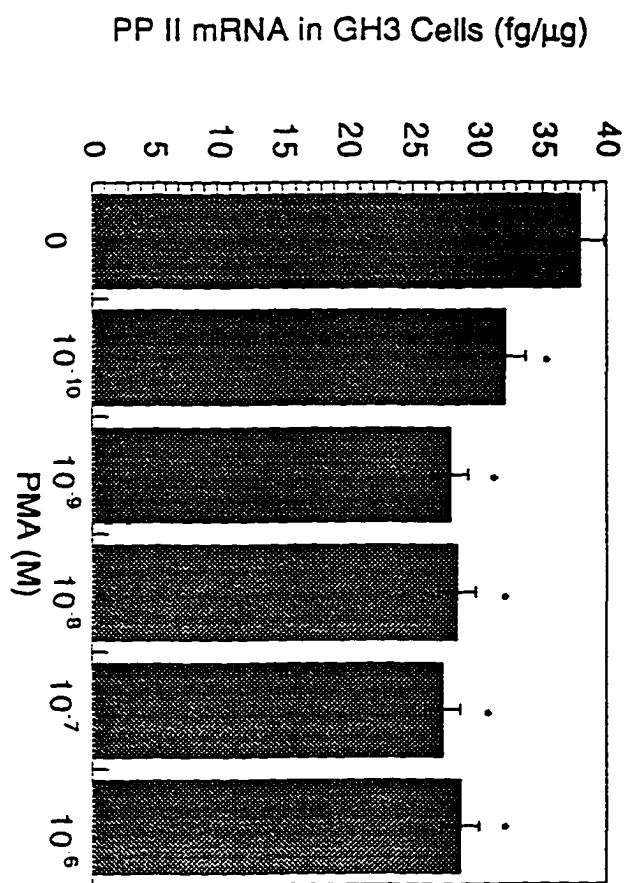


Figure 6

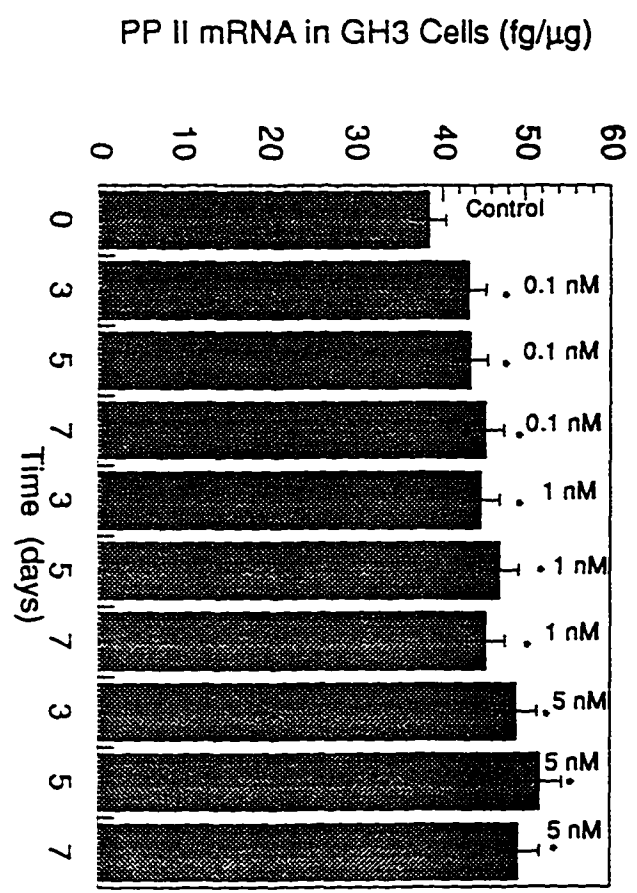


Table 1. Regulation of pyroglutamyl peptidase II mRNA levels in GH3 cells by TRH and PKC activators.

Additions	mRNA (fg/ μ g total RNA) (Mean $\pm$ SE, n=6)	Specific activity (units/mg)
Control	17.8 $\pm$ 0.7	0.114 $\pm$ 0.030
TRH (10^{-7} M)	8.0 $\pm$ 0.5*	0.073 $\pm$ 0.023*
PMA (10^{-7} M)	8.9 $\pm$ 0.3*	0.072 $\pm$ 0.021*
Phorbol 12,13 dibutyrate (10^{-7} M)	9.9 $\pm$ 0.3*	0.076 $\pm$ 0.021*
4 α -TPA (10^{-7} M)	17.2 $\pm$ 0.5	0.117 $\pm$ 0.032
H7 (10^{-5} M)	18.1 $\pm$ 0.8	0.107 $\pm$ 0.033
TRH (10^{-7} M)+ H7 (10^{-5} M)	17.1 $\pm$ 0.7**	0.111 $\pm$ 0.035**
PMA (10^{-7} M) + H7 (10^{-5} M)	17.4 $\pm$ 0.8***	0.113 $\pm$ 0.034***

Pyroglutamyl peptidase II mRNA was quantitated by a solution hybridization ribonuclease protection assay as described in Materials and Methods. GH3 cells were incubated with the tested compounds for 16 h. Pyroglutamyl peptidase II specific activity was determined as described in Materials and Methods in Chapter 2. A unit of enzyme activity is defined as the release of 1 nmol naphthylamine (NA) per hour from the substrate pGlu-His-Pro-NA. Data are mean values of three samples $\pm$ SEM.

* $P < 0.05$ compared to control

** $P < 0.05$ compared to TRH (10^{-7} M)

*** $P < 0.05$ compared to PMA (10^{-7} M)

Table 2. Effect of treatment of GH3 cells with 10^{-7} M T3 for varying periods of time

<u>Time of exposure (h)</u>	<u>mRNA (fg/μg total RNA)</u>	<u>Specific Activity(unit/mg)</u>
0	38.5 ± 2.7	0.10 ± 0.02
0.5	40.7 ± 2.7	0.10 ± 0.02
1	38.7 ± 2.4	0.11 ± 0.02
2	39.7 ± 3.3	0.10 ± 0.02
4	38.8 ± 1.6	0.10 ± 0.02
8	38.6 ± 2.8	0.10 ± 0.02
16	39.9 ± 2.2	0.10 ± 0.02
24	38.2 ± 2.7	0.11 ± 0.02

GH3 cells were exposed to 10^{-7} M T3 for the times indicated and total cytosolic RNA extracted for measurement of pyroglutamyl peptidase II mRNA by a ribonuclease protection assay (Materials and Methods). Results expressed as mean $\pm$ S.E. n = 4 for all time periods.

Chapter 5

Summary

TRH functions as a neurohormone and neurotransmitter in the CNS, and possesses a number of pharmacologically and clinically relevant properties. Its specific degrading enzyme, pyroglutamyl peptidase II provides a synaptosomal mechanism for inactivation of neuronally released TRH.

Pyroglutamyl peptidase II was first discovered in rat serum independently by Taylor and Dixon in 1978 and by Bauer and Nowak (1979). Biochemical characterization differentiated it from the known enzyme pyroglutamyl peptidase I. The early literature termed this enzyme thyroliberinase because of its high substrate specificity. The same enzymatic activity was later characterized in the central nervous system. In this laboratory, the existence of pyroglutamyl peptidase II in CNS was successfully delineated by the application of two specific inhibitors of the TRH-degrading enzymes pyroglutamyl peptidase I and prolyl endopeptidase. Regional distribution revealed that pyroglutamyl peptidase II activity is predominantly present in the brain. A significant amount of enzyme activity was also found in lung and pituitary. In brain, the distribution of enzyme activity is uneven, with highest activity in cortex (Friedman and Wilk, 1986).

The biochemical characterization of brain pyroglutamyl peptidase II leaves little doubt that the brain enzyme is of the same identity as serum thyroliberinase. Wilk and Wilk (1989) confirmed the substrate specificity by the use of synthetic and natural peptides. TRH is the only known naturally occurring substrate of pyroglutamyl peptidase II (Wilk and Wilk, 1989). The enzyme is sensitive to metal chelating agents (Wilk and Wilk, 1989) and is now classified as a member of the Zinc-dependent metalloproteinase family (Czekay and Bauer, 1993).

In this laboratory, specific inhibitors of pyroglutamyl peptidase I, pyroglutamyl peptidase II and prolyl endopeptidase have been developed and applied to evaluate the functional aspect of these enzymes in the turnover of neuronally released TRH. Only inhibition of pyroglutamyl peptidase II leads to an increased recovery of TRH released from brain slices by K⁺ stimulation. Inhibition of pyroglutamyl peptidase I, prolyl endopeptidase or their combination has no noticeable effects on recovery of released TRH (Charli, et al, 1989).

Pyroglutamyl peptidase II is a regulated enzyme. Serum thyroliberinase activity is significantly reduced by hypothyroidism and increased by hyperthyroidism (White, et al, 1976). Adenohypophyseal pyroglutamyl peptidase II is also strigently regulated by thyroid hormone (Bauer, 1987). Suen and Wilk showed that acute administration of thyroid hormone to rats incresed pyroglutamyl peptidase II activity in pituitary and frontal cortex (Suen and Wilk 1989). As a component of the TRH transmission system, pyroglutamyl peptidase II is also regulated by TRH. In primary cultures of rat adenohypophyseal cells, TRH and phorbol ester were shown to diminish pyroglutamyl peptidase II activity (Vargas, et al, 1994). In Y-79 human retinoblastoma cells, Suen and Wilk demonstrated the inactivation of pyroglutamyl peptidase II activity by the protein kinase C activator phorbol ester (Suen and Wilk, 1990).

Several attempts to purify pyroglutamyl peptidase II both in this laboratory and elsewhere were unable to achieve homogeneity on SDS-PAGE. This had hampered the understanding of the structure of pyroglutamyl peptidase II and prevented the development of antibodies (Wilk and Wilk, 1989; O'Connor and O'Cuinn, 1984, 1985). Our initial aim was to successfully purify

pyroglutamyl peptidase II and thereby obtain information of the amino acid sequence of tryptic peptides for subsequent cDNA cloning. By optimizing the conditions for trypsinization of this membrane-bound enzyme and also optimizing several column chromatographic steps, we have purified pyroglutamyl peptidase II from rabbit brain to apparent homogeneity as judged both by SDS-PAGE and nondissociating gel electrophoresis. We characterized its subunit structure to be a homodimer with a molecular mass of 115 kDa for each subunit. The specific enzymatic activity was estimated as 135 units/mg. Amino acid sequences of tryptic peptides were obtained from the purified enzyme. Immunizing mice with purified enzyme resulted in an antiserum recognizing pyroglutamyl peptidase II. At this stage of studies, we had necessary amino acid sequence information for synthesis of DNA probes for screening a cDNA library, and alternatively an antibody for expressing cloning. However, while the work was progressing, the cDNA cloning of pyroglutamyl peptidase II was published (Schauder et al., 1994), and our studies were in agreement with the cDNA information reported.

There was no information about the brain regional distribution of the mRNA levels of pyroglutamyl peptidase II, and information on the organ distribution of its mRNA was limited (Schauder, et al, 1994). We were interested in furthering our understanding about the distribution and regulation of pyroglutamyl peptidase II mRNA levels in brain regions, pituitary and other organs. To overcome the difficulty of the low abundance of pyroglutamyl peptidase II mRNA, we have established a sensitive, reliable and quantitative solution hybridization ribonuclease protection assay based on the sequence of the recently cloned cDNA. Our studies have documented the uneven distribution of pyroglutamyl peptidase II mRNA levels in rat major organs and

brain regions. The highest level of mRNA of pyroglutamyl peptidase II was found in brain (705 ± 47 fg/ μ g total RNA), which agrees with a role for TRH as a neurotransmitter/neuromodulator in brain. Significant levels of the mRNA were also found in lung (136 ± 13 fg/ μ g) and pituitary (42 ± 2.3 fg/ μ g), whereas liver, heart, duodenum/pancreas, intestine, spleen, kidney, testis, and muscle contained lower but detectable amounts of pyroglutamyl peptidase II mRNA. TRH-like immunoactivities have been detected in peripheral organs, and TRH has been proposed to be a physiological mediator (Prasad, 1987). The existence of its degrading enzyme and mRNA in these peripheral organs supports a potential physiological function of TRH or related peptide in these organs. The relatively abundant level of pyroglutamyl peptidase II activity and mRNA in lung is of particular interest and requires further investigation. Probably, a reasonable next step is to identify the cell type expressing pyroglutamyl peptidase II mRNA by *in situ* hybridization studies. In the brain the distribution of pyroglutamyl peptidase II mRNA is uneven with highest levels found in posterior cortex (1056 ± 83 fg/ μ g) and lowest in brain stem (129 ± 16 fg/ μ g). The regional mRNA distribution correlates with enzymatic activity with the exception of cerebellum. In cerebellum, the mRNA level is relatively high whereas the enzymatic activity is relatively low. More information concerning the location of pyroglutamyl peptidase II in the putative TRH neuronal system would require *in situ* hybridization and immunocytochemical studies.

As pyroglutamyl peptidase II activities in pituitary, frontal cortex, serum, and liver were up-regulated by thyroid hormone (White, et al, 1976; Emerson and Wu, 1987; Bauer, 1987; Suen and Wilk, 1989; Scharfmann, et al, 1990), we sought to examine the potential change of mRNA levels of pyroglutamyl peptidase II, under the same mode of treatment with thyroid hormone as used in

studies measuring enzymatic activity. A single dose of T₃ markedly increased the level of pyroglutamyl peptidase II mRNA in the pituitary ($p < 0.01$). It suggests that in pituitary the regulation of pyroglutamyl peptidase II by thyroid hormone may contribute to the homeostasis of the TRH system in the hypothalamic-pituitary-thyroid axis. The cell type responding to thyroid hormone in pituitary is unclear. The *in situ* hybridization histology of pyroglutamyl peptidase II mRNA, combined with immunocytochemical localization of lactophyte and thyrotrophs before and after the administration of thyroid hormone, would hopefully solve this problem. The source of serum thyroliberinase is unknown. Elevation of pyroglutamyl peptidase II mRNA in liver by T₃ ($p < 0.05$) suggests that this organ is the source of the enzyme in serum. Another potential source is the proteolysis from pituitary cell membranes, where pyroglutamyl peptidase II is upregulated by thyroid hormone. The fact that facile trypsinization can release this enzyme from washed brain pellets supports this notion.

TRH was proposed to possess therapeutic value and has been used clinically for diagnosis and treatment. Specific receptors mediate the action of TRH, which is a G protein-coupled receptor linked to the phospholipase C pathway (Gershengorn, 1990). It was of interest to study the potential regulation of its degrading enzyme after prolonged exposure to TRH. Following a single intravenous administration of 300 µg TRH to rats, we did not detect any significant changes of pyroglutamyl peptidase II mRNA levels in major rat organs or in any brain region. From our studies, we assume that there is no significant change of pyroglutamyl peptidase II expression level following a pharmacological dose of TRH.

We were interested in establishing the GH3 cell as a model system for studying pyroglutamyl peptidase II mRNA regulation and exploring the underlining mechanisms. The GH3 cell is a well characterized prolactin and growth hormone secreting rat pituitary tumor cell line. It contains functional TRH receptors (Aragay, et al, 1992), pyroglutamyl peptidase II (Wilk, et al, 1988), and thyroid hormone receptors (Petty, 1995). We sought to determine the steady state mRNA levels before and after treatment with thyroid hormone, TRH, and epidermal growth factor in GH3 cells. In contrast to the *in vivo* studies, exposure to T3 at concentrations of 10^{-10} to 10^{-6} M, and from 2 hr to 24 hr did not change pyroglutamyl peptidase II mRNA levels. These experiments revealed that the fundamental mechanisms regulating pyroglutamyl peptidase II biosynthesis are different in GH3 cells and the pituitary cells that respond to thyroid hormone.

TRH decreased pyroglutamyl peptidase II mRNA with a maximal effect after 16h exposure to 10^{-7} M TRH. The protein kinase C (PKC) activators 1,12-O-tetradecanoyl phorbol-13-acetate (PMA), and phorbol-12,13-dibutyrate also decreased pyroglutamyl peptidase II mRNA levels, whereas the inactive phorbol analog 4- α -TPA had no effect. The protein kinase inhibitor H-7 inhibited the decrease of pyroglutamyl peptidase II mRNA by TRH and PMA. We concluded that the effect of TRH on pyroglutamyl peptidase II mRNA was at least in part via the PKC activating pathway. The down-regulation of pyroglutamyl peptidase II mRNA by TRH may provide a positive feedback mechanism for the action of TRH on pituitary lactotrophs. Taking the results of thyroid hormone and TRH *in vivo* and *in vitro* together, it seems possible that in the pituitary, the thyrotroph is primarily regulated by thyroid hormone while the lactotroph is regulated by TRH.

It has been proposed that in the pituitary, pyroglutamyl peptidase II is located preferentially on lactropic cells (Bauer, et al, 1990). Epidermal growth factor (EGF) has been shown to remarkably increase the population of prolactin-secreting cell types in GH3 cultures (Felix, et al, 1995). If indeed pyroglutamyl peptidase II is preferentially located on prolactin secreting cells, the shift to a prolactin secreting cell population may result in an increase in pyroglutamyl peptidase II. EGF also modulates TRH receptors in pituitary cloned cells (Hinkle, et al, 1991) and may therefore also have a regulatory effect on pyroglutamyl peptidase II. Our experiments demonstrated that EGF treatment produced a small but significant increase in pyroglutamyl peptidase II mRNA in GH3 cells. The maximal effect was a 30% increase in pyroglutamyl peptidase II mRNA, observed following treatment with 5 nM EGF. Our observations with EGF suggest a modulatory role of EGF on pyroglutamyl peptidase II.

In summary, our recent studies on pyroglutamyl peptidase II have established the purification and subunit structure of this enzyme, demonstrated the regional distribution and regulation of its mRNA in rats and in GH3 cells. Based on the tools developed and information we have obtained, the in situ hybridization and immunocytochemical histology can be pursued to localize in more detail the distribution of pyroglutamyl peptidase II. Site-directed mutagenesis studies on the active and regulatory elements of its cDNA can be expected. The further elucidation of the structure and function of this enzyme may be provided by the transgenic approaches. Specific inhibitors may be needed as a pharmacological alternative to TRH analogs.

Chapter 6

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